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PLANT PHYSIOLOGY

VOLUME 31 JANUARY, 1956 NUMBER 1

FREE RIBONUCLEIC ACID PARTICLES IN HOMOGENATES OF PLANT TISSUE[1,2,3]

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In a study of isolation methods for plant viruses, an extraction buffer was found which permitted the partial purification, by differential centrifugation, of an unstable fruit tree virus from cucumber leaf tissue (2). In applying this procedure to apparently healthy plant tissue, an unexpected number of high particle weight electrophoretic components was found. Some of the electrophoretic components were apparently composed solely of ribonucleic acids, and, as far as we know, this was the first record of the separation of high particle weight nucleic acids from plant cytoplasm.

METHODS

The isolation procedure consisted of homogenizing one part of fresh leaf tissue (25 gm) with 3 parts of extraction buffer (75 ml). All operations were carried out at about 4° C. The extraction buffer was composed of 0.007 M potassium phosphate, 0.007 M ethylene diamine tetraacetic acid, 0.01 M cysteine hydrochloride, 0.01 M sodium diethyl barbiturate, and sufficient potassium hydroxide to bring to pH 7.8. The homogenate was filtered with suction through a thin pad of glass wool to remove coarser cell debris, and then centrifuged at 15,000 rpm for 15 min (20,360 × g maximum) in a Spinco preparative centrifuge (No. 40 head) to remove the finer cell debris. The high particle weight proteins and nucleic acids were separated at 40,000 rpm for 120 min (144,700 × g maximum) and the supernatant containing the lower weight materials was discarded. The pellets were dissolved with 2 ml of pH 7.0 buffer (composed of 0.02 M neutral potassium phosphate and 0.15 M potassium chloride) and combined in a single tube. The low and high speed centrifugation cycles were repeated, yielding a clear gelatinous pellet. This pellet was dissolved with 3 ml of phosphate-chloride buffer and the debris removed at 5,000 rpm for 15 min (2,264 × g maximum). The colorless (sometimes with a tinge of green), slightly opalescent solution was subjected directly to electrophoretic analysis in a Perkin-Elmer electrophoresis apparatus. The phosphate-chloride buffer was used during electrophoresis as in the isolation procedure, so that it was not necessary to dialyze the sample. Concentrations of the various electrophoretic components were calculated from the areas under the peaks.

All data reported in this paper are from plants grown in an insect-free greenhouse in pasteurized soil. A number of varieties were used. Typical varieties chosen for this report included cucumber, *Cucumis sativus* L. var. Chicago Pickling; tomato, *Lycopersicum esculentum* Mill. var. Early Wonder; cowpea, *Vigna sinensis* (Torner) Savi; bean, *Phaseolus vulgaris* L. var. Stringless Green Pod; mahaleb cherry, *Prunus mahaleb* L.; and tobacco, *Nicotiana Tabacum* L. var. Turkish and *N. rustica* L.

RESULTS

Typical results from electrophoresis studies are shown in figure 1. Ultraviolet absorption spectra of successive increments of both ascending and descending limbs were made. The descending limb yielded typical nucleoprotein spectra throughout. The ascending limb, on the other hand, yielded nucleic acid spectra until the position of the principal protein peak (P_3) was reached, when nucleoprotein spectra were obtained (fig 2). The slower moving components were thus considered to be nucleoproteins and were arbitrarily designated P_1, P_2, and P_3. The faster moving components were considered to be nucleic acids and were arbitrarily designated N_1, N_2, N_3, and N_4. The principal nucleoprotein component (P_3) is similar if not identical to the fraction I protein of Wildman and Bonner (6) and the nucleoprotein isolated by Pirie (5).

The electrophoretic mobilities of the various high-weight components are shown in table I. It can be seen from table I and figure 1 that the cytoplasmic components are somewhat similar even though the

[1] Received March 21, 1955.

[2] Scientific Paper No. 1300, Washington Agricultural Experiment Stations, Pullman, Washington. Project No. 865.

[3] These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and the State College of Washington (NR 134-121), and were supported in part by funds provided for biological and medical research by the State of Washington Initiative Measure No. 171.

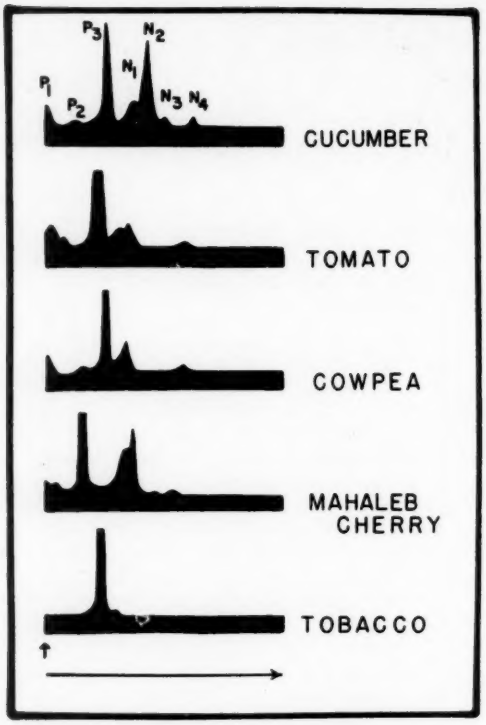

FIG. 1. Electrophoretic patterns of high-weight proteins and free nucleic acids from leaves of various plant species. Ascending limb patterns at 60 minutes. P_1, P_2, and P_3 are arbitrarily lettered nucleoproteins and N_1, N_2, N_3, and N_4 are arbitrarily lettered free nucleic acids.

plant species are varied. Tobacco leaves yield the lowest number of components, but with tobacco, as with the other plants tested, large amounts of free ribonucleic acid may be recovered if immature leaves and growing tips are used.

The free nucleic acids are more mobile in the electrophoresis cell and can thus be drawn off from the ascending limb as a mixture ($N_1 - N_4$) and separated from the nucleoproteins. When thus separated, the free nucleic acids give negative Biuret and Ninhydrin tests for proteins. On hydrolysis with *N* sulfuric acid, ribose can readily be detected in them, either with an orcinol color test or by paper chromatography using a phenol-water solvent and developing the spot with ammoniacal silver nitrate by the method of Partridge and Estall (4). No evidence of desoxyribose could be detected in the free ribonucleic acids.

Sufficient free nucleic acids were obtained from immature leaves to permit hydrolysis with perchloric acid by the method of Marshak and Vogel (3) and the determination of purine and pyrimidine composition by paper chromatography using the isopropanol solvent of Wyatt (7). Typical results of such analyses are shown in table II. Only adenine, guanine, cytosine, and uracil could be detected in the free ribonucleic acids confirming the other observations that these constituents are composed solely of ribonucleic acid (RNA). Furthermore, when chromatograms of the free ribonucleic acids were sprayed with ninhydrin, only a trace of color developed. On the other hand, when nucleoprotein chromatograms were sprayed with ninhydrin an intense color developed. Thus, if proteins were present in the free RNA, they were present only in trace amounts.

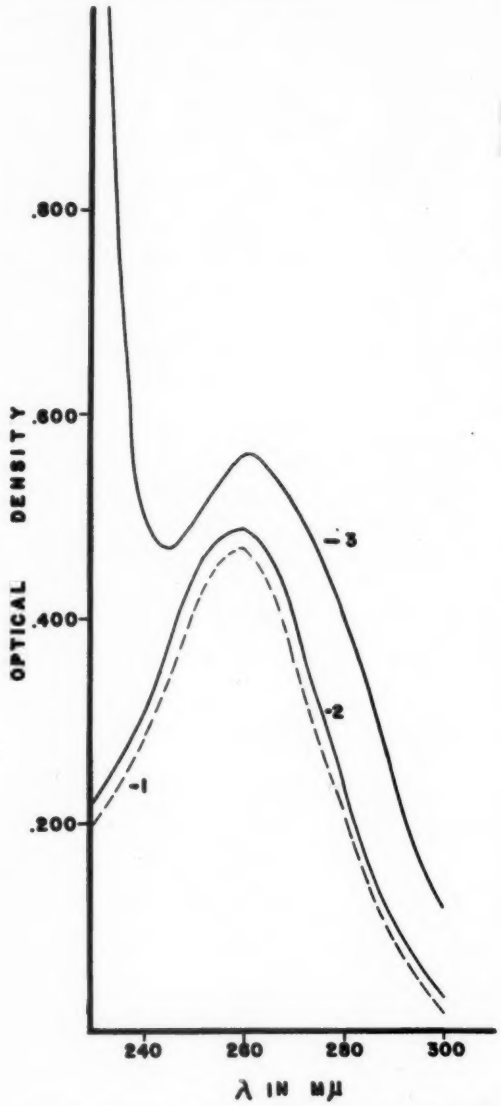

FIG. 2. Ultraviolet absorption spectra of 1) free ribonucleic acids from cucumber (N_1, N_2, N_3, and N_4); 2) commercial ribonucleic acid from yeast; and 3) nucleoprotein isolates from cucumber (P_1, P_2, and P_3).

TABLE I

ELECTROPHORETIC MOBILITIES OF HIGH-WEIGHT NUCLEOPROTEINS AND FREE NUCLEIC ACIDS FROM LEAF CYTOPLASM OF VARIOUS PLANTS

PLANT	COMPONENTS						
	NUCLEOPROTEINS			FREE NUCLEIC ACIDS			
	P_1	P_2	P_3	N_1	N_2	N_3	N_4
	Electrophoretic mobility $\times 10^{-5}$ *						
Cucumber	0.4	3.3	5.5	7.3	8.6	11.8	14.1
Tomato	0.5	2.5	4.2	6.9	7.7	...	14.2
Cowpea	0.7	3.5	6.0	7.4	8.4	...	15.0
Mahaleb cherry ..	0.4	0.9	3.6	6.5	8.1	11.6	14.1
Tobacco	..	..	4.9	7.0	..	...	...

* In buffer composed of 0.02 M neutral potassium phosphate plus 0.15 M KCl.

It should be noted that the data in table II are from mixtures of electrophoretic components. However, the free RNA differs significantly in concentration of one or more bases from the mixture of free RNA plus protein RNA, indicating that the free RNA and the RNA combined with the protein are not the same. Therefore it is not likely that the free RNA is derived directly from the high-weight nucleoprotein RNA.

It will be noted that the concentration of the guanine plus cytosine pair is greater in all cases than the adenine plus uracil pair. This is in accord with the work of Elson and Chargaff (1) who report a preponderance of guanine plus cytosine in all RNA (except yeast) studied in their laboratory, while the adenine plus thymine pair predominates in deoxyribonucleic acids. Most of their work was done on nucleic acids from animal sources.

The question of whether the free nucleic acids occur as such in intact cytoplasm or whether they represent breakdown or polymerized products from the isolation procedure is difficult to resolve. It was thought that a study of the distribution of the free nucleic acids in various parts of the plant might aid in answering this question. With this in view, cucumber seeds were germinated in the dark. The roots, hypocotyls, and cotyledons were separated and analyzed as previously indicated. The roots yielded only 55 ppm of the fresh weight of nucleoproteins and no detectable free nucleic acids; the hypocotyls 40 ppm nucleoproteins and 60 ppm free nucleic acids; and the cotyledons and growing point 1060 ppm nucleoproteins and 2335 ppm free nucleic acids.

TABLE III

NUCLEOPROTEIN AND FREE NUCLEIC ACID CONCENTRATION (PPM OF THE FRESH WT) IN LEAVES OF DIFFERENT AGES FROM 5-WEEK-OLD CUCUMBER PLANTS

LEAF	NUCLEOPROTEIN (PPM)	FREE NUCLEIC ACID (PPM)
Apical (1–3)	515	975
4	440	460
5	395	220
6	665	240
7	760	190
Basal (8)	480	75
Leaf 8 (4 weeks earlier)	940	565

Another series of cucumber plants were grown in the green house. The first mature leaf was analyzed and four weeks later all of the leaves were analyzed. The results are shown in table III. The amount of high-weight free nucleic acid varies markedly with leaf age. The highest concentration occurs in the young leaves; and, as the leaves mature, the free nucleic acid content decreases.

Cucumber cotyledons usually persist on the plant for several weeks, after which time they turn yellow

TABLE II

MOLAR RATIOS OF PURINE AND PYRIMIDINE BASES IN HIGH-WEIGHT FREE NUCLEIC ACIDS AND NUCLEOPROTEINS *

SOURCE	BASE				RATIO
	ADENINE	URACIL	GUANINE	CYTOSINE	$\frac{\text{ADENINE + URACIL}}{\text{GUANINE + CYTOSINE}}$
Cucumber					
Free RNA (N_1, N_2, N_3, N_4) ...	26	18	32	24	0.78
Free RNA + protein RNA	26	20	34	21	0.84
Tomato					
Free RNA (N_1, N_2, N_4)	17	23	30	30	0.67
Free RNA + protein RNA	24	18	32	25	0.74
Tobacco					
Free RNA (N_1, N_4)	21	22	28	29	0.75
Free RNA + protein RNA	24	19	33	25	0.74
Bean					
Free RNA (N_1, N_2, N_4)	20	22	30	27	0.74
Free RNA + protein RNA	22	21	33	24	0.75
			L.S.D. at 5% level 0.8		

* Total bases = 100.

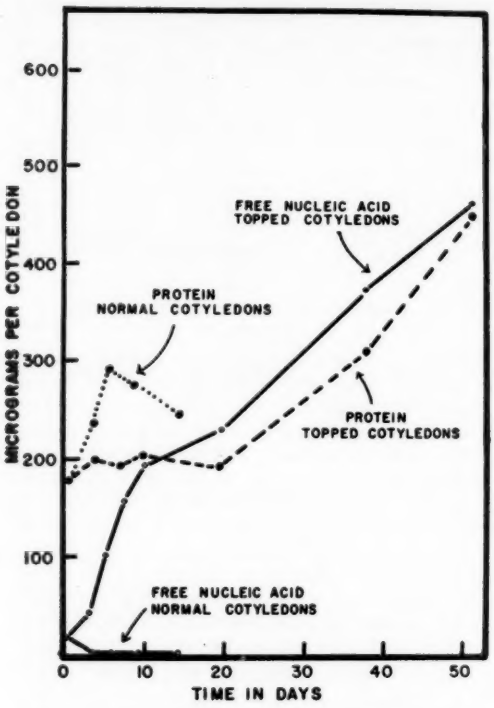

FIG. 3. Changes with time in high-weight cytoplasmic constituents of cucumber cotyledons from intact and de-topped plants.

and dry. If the epicotyl and axillary buds are removed, however, the cotyledons remain green for several months. The high-weight constituents of cotyledons from normal and de-topped plants were compared, and the results are shown in figure 3. The free nucleic acid content of normal cotyledons is very low, but there is a rapid accumulation of free nucleic acids in cotyledons from de-topped plants. After two weeks the free nucleic acid content exceeds the nucleoprotein content.

While not conclusive, the results would indicate that free nucleic acids are common constituents of leaf cytoplasm. The fact that free nucleic acids accumulate to relatively high concentration in cotyledons of de-topped plants suggests that they may serve as a nucleic acid reserve, although they may serve specific functions as well. So far as we know, this is the first time free nucleic acids have been reported, possibly owing in part to the fact that they are very easily lost in the isolation procedure. The free nucleic acids are precipitated by acids and by high salt concentrations.

SUMMARY

High-weight free ribonucleic acids have been found in homogenates of plant tissue from a number of different kinds of plants, and may be isolated by differential ultracentrifugation followed by electrophoresis. At least four different types of free ribonucleic acids may be characterized by their electrophoretic mobility. They are more mobile than accompanying nucleoproteins and may thus be separated by electrophoresis. The free nucleic acids give typical nucleic acid absorption spectra and ribose may be detected in them, after hydrolysis, either with an orcinol color test or by paper chromatography. When the free nucleic acids are hydrolyzed with perchloric acid, only the bases adenine, guanine, cytosine, and uracil could be detected by paper chromatography.

The free nucleic acids are unstable and care must be exercised in their isolation. Their concentration is usually inversely proportional to the age of the tissue, and mostly confined to the above ground parts of plants. If the epicotyl and axillary buds are removed from cucumber seedlings, relatively large amounts of free nucleic acids accumulate in the cotyledons for several weeks. Cotyledons from intact plants contain relatively little free nucleic acid.

It is concluded that free ribonucleic acid is a common constituent of young leaves, buds, and growing points of many kinds of plants. It is not known whether the free nucleic acid merely serves as a reserve or whether it has specific functions.

LITERATURE CITED

1. ELSON, D. and CHARGAFF, E. Regularities in composition of pentose nucleic acids. Nature 173: 1037–1038. 1954.
2. LINDNER, R. C., KIRKPATRICK, HUGH C., and WEEKS, T. E. Some factors affecting the purification of a stone fruit virus. Phytopathology 45: 574–576. 1955.
3. MARSHAK, ALFRED and VOGEL, HENRY J. Microdetermination of purines and pyrimidines in biological materials. Jour. Biol. Chem. 189: 597–605. 1951.
4. PARTRIDGE, S. M. and WESTALL, R. G. Filter-paper partition chromatography of sugars. I. General description of application to the qualitative analysis of sugars in apple juice, egg white, and fetal blood of sheep. Biochem. Jour. 42: 238–250. 1948.
5. PIRIE, N. W. Isolation from normal tobacco leaves of nucleoprotein with some similarity to plant viruses. Biochem. Jour. 47: 614–625. 1950.
6. WILDMAN, S. G. and BONNER, J. Proteins of green leaves. I. Isolation, enzymatic properties, and auxin content of spinach cytoplasmic proteins. Arch. Biochem. 14: 381–413. 1947.
7. WYATT, G. R. Purine and pyrimidine composition of desoxypentose nucleic acids. Biochem. Jour. 48: 584–590. 1951.

EFFECTS OF PETROLEUM OILS ON THE CARBON DIOXIDE OUTPUT IN RESPIRATION OF PARSNIP AND MUSTARD[1,2]

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Herbicidal oils are used extensively for weeding umbelliferous crop plants such as carrots and parsnips, but as yet, a satisfactory explanation has not been given for the selective action of these oils. Investigations were undertaken to determine if the effects of certain petroleum oils on some of the physiological processes of plants would elucidate the mechanism of the selective action. Some of the results have been presented to meetings of scientific workers, and summaries (3, 7) have been published of these presentations. The present paper deals with the effects of herbicidal oils on the respiration of plants.

MATERIALS AND METHODS

The plants used were parsnip, *Pastinaca sativa* L. var. Hollow Crown, and common mustard, *Brassica Kaber* Wheel. var. *pinnatifida* Stokes. These plants were grown at 21° C in a greenhouse with southern exposure and shaded by slats during the summer months. Parsnip was given 35 fc of supplementary incandescent light from 5 to 9 P.M. during the winter months. As mustard leaves are very small when grown under long days the day length for the mustard plants was shortened to 12 hours during the summer months by darkening them with a black cloth, and they were not given supplementary light during the winter months. The ages of the plants used were, parsnip 60 to 70 days and mustard 30 to 40 days from the time of sowing. The leaf areas varied from 40 to 60 cm^2 for parsnip and from 30 to 60 cm^2 for mustard.

The main 2 oils used in this work were a petroleum naphtha, representative of the selective herbicidal oils, and a paraffinic oil, representative of non-phytotoxic oils. The chemical and physical properties of these oils are given in table I. One large sample of each of the oils was used throughout the study and was kept tightly stoppered in a dark place. The petroleum naphtha belongs to that group of oils variously termed stoddard solvent, mineral spirits, solvent naphthas etc. As it is a petroleum oil and its boiling point is in the naphtha range we have chosen to call it a petroleum naphtha.

Other phytotoxic oils used in this study were: (a) the naphthene fraction of a petroleum naphtha, (b) the naphthene-paraffin fraction of a petroleum naphtha, (c) *p*-cymene, an aromatic hydrocarbon, (d) n-dodecane, a paraffinic hydrocarbon, (e) 20 % *p*-cymene in n-dodecane, (f) 300 and 3000 ppm of Hg in the petroleum naphtha and (g) 450 and 3000 ppm of Hg in the paraffinic oil. To obtain the naphthene fraction and the naphthene-paraffin fraction of petroleum naphtha, 2 petroleum naphthas were chosen, one containing naphthenes and aromatics, and the other containing paraffins, naphthenes and aromatics. The aromatics were removed from both of these oils by silica gel percolation leaving (a) a naphthene fraction and (b) a naphthene-paraffin fraction containing 68 % naphthenes and 32 % paraffins by volume. The Hg was the oil-soluble methyl-9-acetoxymercuri-10-methoxystearate, prepared according to the method of Ralston et al (9).

The respiration determinations were made by measuring the CO_2 output of the intact shoots of plants with an infrared microanalyzer. This microanalyzer is similar to the one used by Scarth et al (10), and is sensitive to 1 ppm of CO_2 in CO_2-free air. The plants were placed in respiration chambers in the laboratory the evening previous to an experiment. To assure that the plants were not low in respiratory substrate for the experiment they were illuminated throughout the night with 600 fc of light and supplied moist, atmospheric air at 40 liters per hour. The next morning the chambers were darkened with heavy black paper, and for the experiment they were supplied CO_2-free air at 25 liters per hour and held at 22.5° C ± 0.5° and around 50 % relative humidity. After darkening, the CO_2 output of the plants was followed until steady rates of respiration were established. This required from 1 to 2 hours, at the end of which time treatment was carried out. Treatment consisted of applying oil drops to the upper leaf surfaces until all of the leaves appeared oil-soaked.

The respiration chambers were made from lantern chimneys, 17 cm in height, 12 cm in diameter at the centre, 6 cm in diameter at the top and bottom, and having a volume of 1230 ml. The tops of the chambers were closed by means of thin, flexible three-hole rubber stoppers, each stopper containing an air inlet tube going nearly to the bottom of the chamber, an air outlet tube near the top of the chamber and a permanently installed dropper filled with the oil to be used for treatment of the plant. This permanently installed dropper permitted application of drops of the oil to the dorsal surface of the leaves without introducing laboratory air containing CO_2. The bottom of each chamber was imbedded in paraffin wax, which covered and separated the soil from the respiration chamber. The stems at the base of the plants were surrounded by plasticine to prevent heat injury when the melted wax was poured over the soil. When applied in this manner the wax did not affect the respiration of the plants over the time period studied.

It was necessary in this work to apply the oils directly to the leaves in the plant chamber to deter-

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TABLE I

PHYSICAL AND CHEMICAL PROPERTIES OF THE OILS

OIL	BOILING RANGE	SAYBOLT VISCOSITY AT 99° C	SURFACE TENSION	PARAFFINS	NAPHTHENES	AROMATICS	ACIDS	PEROXIDES
	°C	*sec*	*dynes/cm*	*%*	*%*	*%*	*%*	*%*
Petroleum naphtha	149 to 204	28.4	26.7 at 20.5° C	25	52	21	<0.002	0.0
Paraffinic oil	204 to 260	30.0	27.7 at 21.5° C	100	0	0	<0.002	0.0

mine their immediate effects on the respiration of the leaves. As the vapours of these oils have infrared absorption bands and as the infrared microanalyzer is non-selective, it was necessary to remove these vapours from the air before the air entered the infrared microanalyzer. A series of 6 scrubbers made from 25 × 200 mm test-tubes and each containing 10 ml of Parke-Davis heavy mineral oil completely removed the vapours of petroleum naphtha from runs of 8 hours or longer. The efficiency of the scrubbers was checked at frequent intervals by obtaining no deflections on the infrared microanalyzer when oils were added to a plant chamber not containing plant material and through which CO_2-free air was passing as in the experimental runs.

In each experiment, in order to establish a relationship between the respiration rates of the control and test plants, 3 or more deflections on the infrared microanalyzer for the CO_2 output were taken for each plant before treatment. The ratio of the means of these deflections for each plant gave the expected rate. Oil was then applied to the leaves of the test plant and deflections for both the control and test plants were followed for the duration of the experiment. It was assumed that the expected rate would remain constant throughout the experiment and any variations from this expected rate after the oil was applied were due to the influence of the oil on the leaves. Actually, when the respiration rates of 2 plants, not treated with oil, were followed for 7 hours the standard deviation of the expected rate was ± 7 %.

From 3 to 9 experiments were carried out on each phase of the work and the results from a single representative experiment are presented. The daily mean respiration rates with their standard deviations are given for the control plants in the figure legends. Where the leaf areas were available the respiration rates have been expressed as μl of CO_2 output per hour per dm^2 of leaf area; otherwise they have been expressed as μl of CO_2 per hour per shoot.

To compare the internal temperature of the leaf with the ambient temperature #40 copper-constantan thermocouples were threaded into the leaf tissue. When petroleum naphtha was applied to the leaves in the dark, the temperature of the leaves decreased by only 0.1 to 0.2° C.

To investigate the effect of O_2 supply, the CO_2 output was followed with the plants in an atmosphere of nitrogen. As the anaerobic respiration rates of parsnip were always lower than the aerobic respiration rates, the parsnip shoots were supplied N_2 from the beginning of the experiment, but the oil was not applied to the leaves until 2 hours later when the lower anaerobic respiration rates were well established for both the control and test plants.

EXPERIMENTAL RESULTS

EFFECTS OF PETROLEUM NAPHTHA AND PARAFFINIC OIL: The petroleum naphtha rapidly increased the CO_2 output of both parsnip and mustard (fig 1).

Tests were made to determine if this measured increase was produced by either the evolution of a volatile substance other than CO_2 but with an infrared absorption band, or the expulsion of CO_2-rich air from the intercellular spaces. When soda lime towers were placed in the air line after the plant chamber the application of petroleum naphtha to the plant did not produce a deflection on the microanalyzer. This result proved that CO_2 was responsible for the measured increase. When 0.1 mg of CO_2 was added to the plant chamber (fig 1, "CO_2 introduced") the response was much faster and less prolonged than when the rapidly penetrating petroleum naphtha was applied. This result indicated that the measured response was not due to the expulsion of CO_2-rich air by the entry of oil into the intercellular spaces. It should be pointed out that the 0.1 mg of CO_2 was added over a period of one minute, which was approximately the time required for the oil to be applied and to penetrate the leaves of a plant. For the reasons stated above we believe that the immediate increase measured by the microanalyzer was a true respiration increase.

Figure 2 gives the time course in hours for the respiration of parsnip and mustard plants following the application of petroleum naphtha to the leaves. Following the immediate increases in the respiration rates, the respiration of parsnip, a resistant plant, remained high for 3 or more hours, then began to return to normal and was near normal by 24 hours after application of the oil. Parsnip leaves showed no visible signs of injury. For mustard, a susceptible plant, the respiration rapidly decreased and was down to 15 % by 24 hours after application of the oil. At this time the leaves were dead and only a portion of the main stem was still living. These respiration results for mustard were obtained in January when the

growing conditions for plants in the greenhouse are poor, and they indicate that the plants had some tolerance to the oil. The respiration of mustard plants grown in the greenhouse during the summer months dropped to almost nil by 4 hours after application of the oil and showed no signs of recovery.

Unlike the results for petroleum naphtha the immediate effect of a non-phytotoxic, paraffinic oil was a decrease in respiration (fig 3). Following this decrease, the respiration of both parsnip and mustard increased and remained above normal for 5 or more hours but had returned to around normal by 24 hours after application of the oil, and the leaves showed no visible injury.

Figure 4 compares the effects on the respiration rates of parsnip shoots of varying the quantities of petroleum naphtha and of paraffinic oil placed on the leaves. As the quantities of petroleum naphtha were increased from 0.2 to 1.6 ml of oil per 100 cm^2 of leaf area, increasing translucence from 25 to 100 % of the total leaf area, there was a corresponding increase in the respiration rate of parsnip shoots. But for the paraffinic oil, the smallest quantity of oil used caused the respiration rates to increase to 127 % of expected rate and the addition of larger quantities of the oil had no further effect on the respiration rate.

Effect of Mercury Added to the Two Oils: The results obtained for petroleum naphtha containing 300 ppm of Hg by weight are shown in figure 5. For parsnip, the respiration results were similar to those for petroleum naphtha alone although the increases in respiration were higher. The 300 ppm of Hg in the petroleum naphtha seemed to have no injurious effect on parsnip leaves over that of the petroleum naphtha alone. For mustard, following the immediate increase in respiration, the decline was accelerated by the presence of Hg. When 3000 ppm of Hg in petroleum naphtha was applied to parsnip leaves the respiration immediately increased and then decreased to below normal by one hour and was down to 53 % of expected rate by 24 hours after application of the oil. These leaves showed definite spots of injury.

The effects of Hg in the paraffinic oil on the respiration of parsnip and mustard shoots are given in figure 6. As 300 ppm of Hg in the paraffinic oil did not decrease the respiration of mustard shoots to below normal but 450 ppm of Hg did, the latter concentration was chosen for this study.

When 450 ppm of Hg in paraffinic oil was applied, the effect on parsnip resembled that of the paraffinic oil alone, but on mustard it resembled that of the petroleum naphtha. There was very little visible injury to the treated parsnip leaves but the mustard leaves were completely killed and the slight respiration was from the main stem. When 3000 ppm of Hg in paraffinic oil was used, the effects on both plants resembled that of petroleum naphtha on mustard. The parsnip leaves showed definite visible injury 24 hours after application of the oil; the mustard leaves were completely killed and only a portion of the main stem remained alive.

Effects of Fractions of Petroleum Naphtha: The effects of a naphthene fraction and a naphthene-paraffin fraction of petroleum naphtha on the respiration of parsnip and mustard shoots are given in figure 7. Both fractions increased the respiration of parsnip and mustard. Their effect, therefore, did not differ from that of the petroleum naphtha containing naphthenes, paraffins and aromatics.

Effects of Pure Hydrocarbons: Table II gives the effects of 2 pure hydrocarbons on the respiration rates of parsnip and mustard shoots. The hydrocarbon n-dodecane is a 12-carbon paraffin boiling at 216° C and *p*-cymene is a 10-carbon aromatic boiling at 175° C. These two hydrocarbons were chosen as being representative of the paraffins and aromatics contained in a petroleum naphtha (boiling range 149° to 204° C).

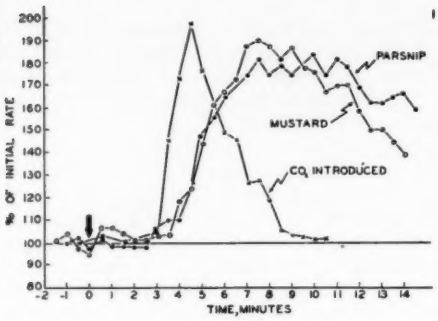
Both the n-dodecane and the 20 % *p*-cymene in n-dodecane had the same effects on respiration of parsnip and mustard as petroleum naphtha. They also acted selectively, causing no injury to parsnip leaves, but completely killing mustard leaves. The *p*-cymene alone caused immediate, temporary increases in the respiration of both parsnip and mustard, followed by decreases to almost nil by 24 hours when the leaves of both species were dead.

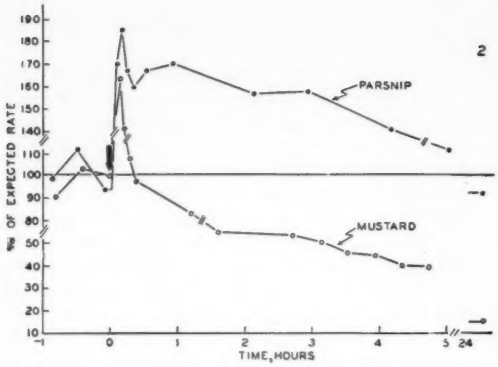
TABLE II

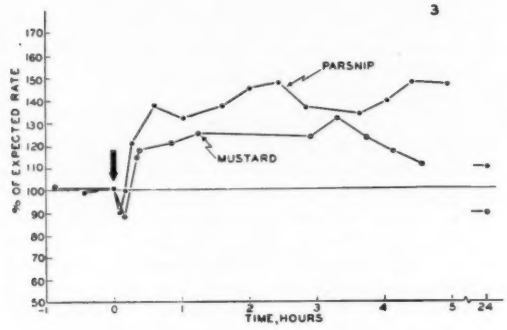
EFFECTS OF n-DODECANE, *p*-CYMENE AND 20 % *p*-CYMENE IN n-DODECANE ON THE RESPIRATION OF PARSNIP AND MUSTARD SHOOTS

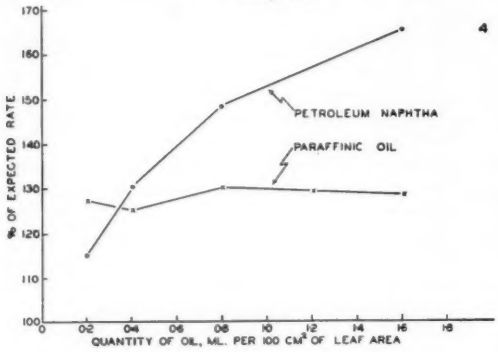
TYPE OF OIL	PARSNIP				MUSTARD			
	TIME FROM TREATMENT, HRS				TIME FROM TREATMENT, HRS			
	½	1	5	24	½	1	5	24
	% expected rate *							
n-Dodecane	138	147	125	110	126	135	80	10
p-Cymene	226	55	21	21	172	61	14	5
20 % *p*-cymene in n-dodecane	236	140	130	101	183	126	47	7

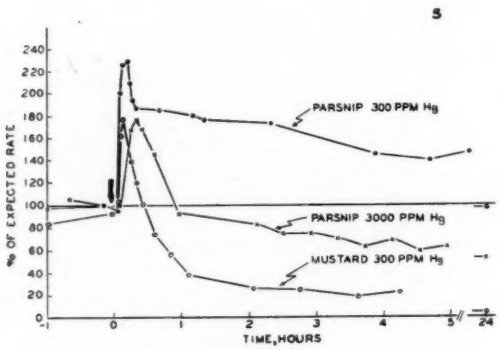
* Expected rate is the ratio of the mean respiration rates for the control and test plants before oil was applied to the test plant. Results are from single representative experiments. Daily mean respiration rates for control plants; parsnip 474 ± 35 μl CO_2/hr × dm^3, mustard 210 ± 53 μl CO_2/hr × shoot.

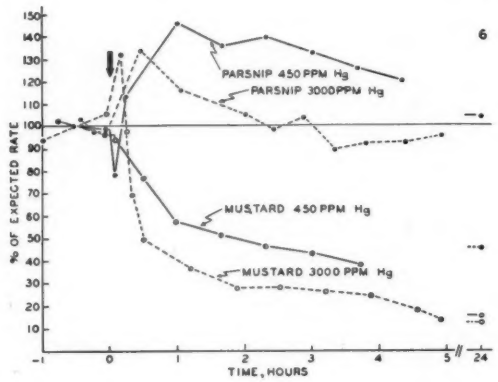

1
% OF INITIAL RATE
PARSNIP
MUSTARD
CO2 INTRODUCED
TIME, MINUTES

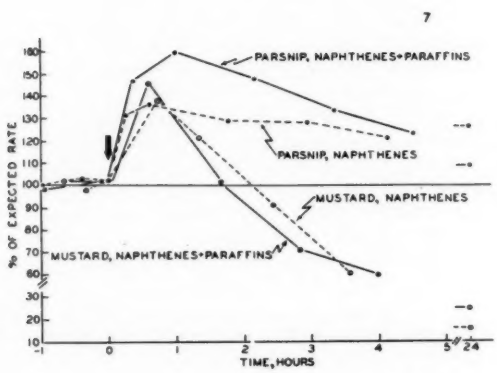

2
% OF EXPECTED RATE
PARSNIP
MUSTARD
TIME, HOURS

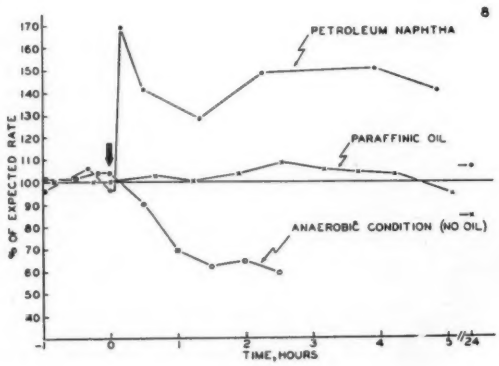

3
% OF EXPECTED RATE
PARSNIP
MUSTARD
TIME, HOURS


4
% OF EXPECTED RATE
PETROLEUM NAPHTHA
PARAFFINIC OIL
QUANTITY OF OIL, ML. PER 100 CM² OF LEAF AREA


5
% OF EXPECTED RATE
PARSNIP 300 PPM Hg
PARSNIP 3000 PPM Hg
MUSTARD 300 PPM Hg
TIME, HOURS


6
% OF EXPECTED RATE
PARSNIP 450 PPM Hg
PARSNIP 3000PPM Hg
MUSTARD 450 PPM Hg
MUSTARD 3000 PPM Hg
TIME, HOURS


7
% OF EXPECTED RATE
PARSNIP, NAPHTHENES+PARAFFINS
PARSNIP, NAPHTHENES
MUSTARD, NAPHTHENES
MUSTARD, NAPHTHENES+PARAFFINS
TIME, HOURS


8
% OF EXPECTED RATE
PETROLEUM NAPHTHA
PARAFFINIC OIL
ANAEROBIC CONDITION (NO OIL)
TIME, HOURS

Effects under Anaerobic Conditions: The presence of oil in the leaves might reduce the O_2 supply to the leaf cells. Figure 8 shows that when parsnip leaves, not treated with oil, were deprived of O_2 by supplying them with N_2 the CO_2 output of the leaves decreased. Therefore, the increases in CO_2 output, observed when oil was applied to parsnip leaves, cannot be attributed to a reduction of O_2 supply to the leaf cells, caused by the presence of oil films on the cell walls.

The effects of petroleum naphtha on the anaerobic respiration of parsnip were similar to those for petroleum naphtha on the aerobic respiration (fig 8). The paraffinic oil had very little if any effect on the anaerobic respiration of parsnip shoots.

Discussion

Although the increase in respiration rate produced by petroleum naphthas in this study agrees with some results reported for other oils (2, 6) it does not agree with the results reported by the majority of workers (1, 4, 5, 8, 11). These workers have reported that the application of a petroleum oil decreased rather than increased the rate of respiration. In those studies reporting decreases in respiration rates closed systems were employed, which would hold oil vapours in close proximity to the leaf tissue. In the present study, unless tissues were actually killed by the oil no indications have been obtained of a decrease in respiration following the application of petroleum naphtha to the leaves. Young and Morris (12) proposed that oils may have 2 effects: (a) a physical effect, plugging stomata, intercellular spaces and tracheae, causing decreases in respiration, and (b) a chemical effect, in which non-lethal toxic chemicals such as unsaturated hydrocarbons increase respiration. From the results presented here there appear to be the following 4 different effects of oils on the respiration of plants.

Decreased Respiration Rates Due to Physical Effect of the Oil: In this study, the only physical effect of oils on respiration comparable to that described by Young and Morris (12) was the immediate, temporary decrease in CO_2 output lasting for only a few minutes when the paraffinic oil was applied to leaves (fig 3). This decrease was likely due to the paraffinic oil interfering with the gas exchange of the leaves when the oil was penetrating the stomata and spreading through the intercellular spaces.

As the viscosity and surface tension of the petroleum naphtha were similar to those of the paraffinic oil, table I, it would be expected that the petroleum naphtha also would cause an immediate temporary decrease in CO_2 output. Actually, there was an immediate increase in CO_2 output when petroleum naphtha was applied to parsnip and mustard leaves. An explanation for the apparent absence of this physical effect of the petroleum naphtha on the leaves can be found in figure 6. The presence of 3000 ppm of

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Fig. 1. Immediate effects of petroleum naphtha on the respiration of parsnip and mustard shoots. Oil applied at arrow. For curve marked "CO_2 introduced" 0.1 mg of CO_2 was introduced at the arrow into the chamber containing a normal parsnip plant. Initial respiration rates for parsnip 216 ± 4 μl CO_2/hr × shoot, for mustard 176 ± 6 μl CO_2/hr × shoot.

Fig. 2. Further effects of petroleum naphtha on the respiration of parsnip and mustard shoots. Expected rate is the ratio of the mean respiration rates for the control and test plants before oil was applied to the test plant. Oil applied at arrow. Daily mean respiration rates for control plants: parsnip 429 ± 32 μl CO_2/hr × dm^2, mustard 126 ± 13 μl CO_2/hr × shoot.

Fig. 3. Effects of a non-phytotoxic, paraffinic oil on the respiration of parsnip and mustard shoots. Oil applied at arrow. Daily mean respiration rates for control plants: parsnip 155 ± 13 μl CO_2/hr × shoot, mustard 174 ± 14 μl CO_2/hr × shoot.

Fig. 4. Effects of varying the quantities of petroleum naphtha and paraffinic oil placed in the leaves on the respiration rates of parsnip shoots. Each point is the mean of 3 to 5 determinations made between 1 to 5 hours after application of the oils. Daily mean respiration rate for control parsnip plant 478 ± 45 μl CO_2/hr × dm^2.

Fig. 5. Effects of petroleum naphtha containing 300 or 3000 ppm of Hg on the respiration of parsnip and mustard shoots. "Parsnip 300 ppm Hg" indicates that 300 ppm of Hg by weight in petroleum naphtha was applied to parsnip. Oils applied at arrow. Note the change in scale for % of expected rate. Daily mean respiration rates for control plants: parsnip 151 ± 13 μl CO_2/hr × shoot, mustard 314 ± 10 μl CO_2/hr × shoot.

Fig. 6. Effects of paraffinic oil containing 450 or 3000 ppm of Hg on the respiration of parsnip and mustard shoots. "Parsnip 450 ppm Hg" indicates that paraffinic oil containing 450 ppm of Hg by weight was applied to parsnip. Oils applied at arrow. Daily mean respiration rates for control plants: parsnip 232 ± 10 μl CO_2/hr × shoot, mustard 492 ± 31 μl CO_2/hr × dm^2.

Fig. 7. Effects of the naphthene fraction and the naphthene-paraffin fraction of petroleum naphtha on the respiration of parsnip and mustard shoots. "Parsnip, naphthenes + paraffins" indicates that the naphthene-paraffin fraction of petroleum naphtha was applied to parsnip. Oils applied at arrow. Daily mean respiration rates for control plants: parsnip 191 ± 31 μl CO_2/hr × shoot, mustard 326 ± 52 μl CO_2/hr × shoot.

Fig. 8. Effects of petroleum naphtha and paraffinic oil on the anaerobic respiration of parsnip shoots. N_2 was supplied for 2 hours before the oils were applied. Oils applied at arrow. For curve marked "anaerobic condition (no oil)" the air supply to the chamber containing a normal parsnip shoot was changed from CO_2 free air to N_2 at the arrow. Respiration rate of parsnip in CO_2 free air 520 ± 23 μl CO_2/hr × dm^2, in N_2 331 ± 20 μl CO_2/hr × dm^2. Daily mean respiration rate for control parsnip in N_2 263 ± 29 μl CO_2/hr × shoot.

Hg in the paraffinic oil resulted in an immediate increase in CO_2 output for both parsnip and mustard with no evidence of an immediate decrease. This indicates that if the toxicity of the oil is high, the increased CO_2 output masks the decrease due to the physical effect of the oil on the leaves.

Increased Respiration Rates under Aerobic Conditions Only: When the paraffinic oil was applied to parsnip and mustard leaves under aerobic conditions (fig 3), a definite increase in respiration was apparent 20 minutes after application of the oil and persisted for 5 hours or longer. As the respiration rates were near normal again by 24 hours, this stimulation of aerobic respiration was only temporary and caused no permanent injury to the leaf tissues. The paraffinic oil had no effect on the respiration of parsnip leaves in an atmosphere of N_2 (fig 8). As the anaerobic conditions would eliminate the aerobic oxidation processes of respiration these results indicate that the paraffinic oil must have stimulated the aerobic oxidation processes. Audus (1), by following CO_2 output, obtained respiration results similar to those above when he gently squeezed cherry laurel leaves between 2 rollers. He found that in an atmosphere of N_2 there was no effect on respiration while in an atmosphere of air the respiration increased. Possibly the stimulation by the paraffinic oil was similar to the mechanical stimulation observed by Audus.

Increased Respiration Rates under Both Aerobic and Anaerobic Conditions: When petroleum naphtha was applied to parsnip leaves there were increases in respiration under both aerobic and anaerobic conditions (figs 2 and 8). The higher respiration rates under aerobic conditions, as compared to those under anaerobic conditions where the aerobic oxidation processes would not function, indicate that both the oxidation and glycolytic processes of respiration were stimulated by petroleum naphtha.

Evidence that stimulation of respiration is a direct chemical effect of some constituent(s) of petroleum naphtha is apparent from the results in figure 4, where the respiration rates of parsnip leaves increased as the quantity of oil placed in the leaves was increased, and also from the results in figure 5 where increasing the toxicity of the oil by adding 300 ppm of Hg caused the respiration rates of parsnip leaves to be higher than when petroleum naphtha alone was used (fig 2). The stimulation of respiration cannot be attributed to acids and peroxides in the oils, because the analyses showed them to be present in negligible amounts, table I; but the results presented in table II and figure 7 indicate that this stimulation may be due to aromatics, naphthenes and/or certain paraffins.

Decreased Respiration Rates Due to Killing of Tissues: The killing of plant tissues is dependent upon their relative tolerance to the phytotoxicity of the oils. Parsnip, a resistant plant, will tolerate moderately toxic oils such as the petroleum naphtha or 20 % *p*-cymene in n-dodecane, while mustard, a susceptible plant is killed by these oils, figure 2 and table II. Parsnip, however, will not tolerate a pure aromatic hydrocarbon such as *p*-cymene.

Even when the plant tissue is about to be killed by an oil, there is an immediate temporary increase in respiration rates. If the respiration rates drop to below normal following this immediate temporary increase, they never return to normal again and the plant tissue is permanently damaged.

Mechanism of Selective Action: An explanation of the selective action of the petroleum naphtha on parsnip and mustard plants is not readily apparent from these respiration studies. The following conclusions, however, may be drawn from the respiration results: (a) Oils that increased respiration rates under aerobic conditions only were non-phytotoxic. (b) Oils that increased respiration rates under both aerobic and anaerobic conditions were either moderately toxic selective herbicidal oils, or they were highly toxic non-selective herbicidal oils. (c) If they were selective herbicidal oils, the high respiration rates persisted 5 hours or longer for parsnip, a resistant plant, the respiration of mustard, a susceptible plant, rapidly declined to below normal within the first hour and was approaching zero within 24 hours. (d) If they were non-selective herbicidal oils, the respiration rates increased immediately after application of the oil but the respiration rapidly declined to below normal within the first hour and the plants were killed within 24 hours.

Summary

As part of a study of the phytotoxic action of petroleum oils, the effects of several oils on the respiration of a resistant plant (parsnip) and of a susceptible plant (mustard) were determined by means of an infrared microanalyzer.

When a non-herbicidal, white, paraffinic oil of boiling range 204 to 260° C and containing no aromatic hydrocarbons was applied to either plant there was a sudden, slight decrease of short duration in respiration, followed by an immediate increase and a gradual return to normal by 24 hours, with no apparent injury.

The herbicidal oils included (a) a petroleum naphtha of boiling range 149 to 204° C and containing 21 % aromatic hydrocarbons, (b) its naphthene fraction, (c) its naphthene-paraffin fraction, (d) a pure paraffin, n-dodecane, (e) 20 % *p*-cymene, an aromatic hydrocarbon, in n-dodecane, (f) 300 ppm of Hg, in the form of methyl-9-acetoxymercuri-10-methoxystearate, in the petroleum naphtha, and (g) 450 ppm of Hg in the paraffinic oil. When applied to parsnip they all produced a sharp rise in respiration followed by a gradual return to normal by 24 hours with no visible injury; but when applied to mustard a similar sharp rise in respiration was followed by a marked reduction and death of the plant.

A concentration of 3000 ppm of Hg in either the petroleum naphtha or the paraffinic oil produced a sharp rise in respiration of parsnip and mustard, followed by a marked reduction and death of the plants.

The sharp rise immediately following the application of these oils was shown to be a true respiration increase and was due neither to the evolution of a volatile substance with an infrared absorption band nor to the expulsion of CO_2-rich air by the entry of oil into the intercellular spaces.

Under anaerobic conditions, the non-herbicidal, white, paraffinic oil had no effect on the respiration of parsnip leaves while the petroleum naphtha caused an increase, indicating that the paraffinic oil stimulated the aerobic oxidation processes while the petroleum naphtha stimulated both the aerobic oxidation processes and the glycolytic processes of respiration.

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Literature Cited

1. Audus, L. J. Mechanical stimulation and respiration of the green leaf. III. The effect of stimulation on the rate of fermentation. New Phytologist 39: 65–74. 1940.
2. Green, J. R. Effect of petroleum oils on the respiration of bean plants, apple twigs and leaves, and barley seedlings. Plant Physiol. 11: 101–113. 1936.
3. Helson, V. A. and Minshall, W. H. The effects of herbicidal oils on the photosynthesis, respiration and transpiration of parsnip and mustard. Abstracts, Amer. Soc. Plant Physiol. Pp. 9–10. 25th Annual Meeting, Columbus, Ohio. 1950; P. 21. 27th Annual Meeting, Ithaca, New York. 1952. (Mimeographed.)
4. Johnson, C. M. and Hoskins, W. M. The relation of acids and peroxides in spray oils to the respiration of sprayed bean leaves and the development of injury. Plant Physiol. 27: 507–525. 1952.
5. Kelley, V. W. Effect of certain hydrocarbon oils on the respiration of foliage and dormant twigs of the the apple. Agr. Expt. Sta., Illinois, Bull. 348: 369–406. 1930.
6. Knight, H., Chamberlin, J. C., and Samuels, C. D. On some limiting factors in the use of saturated petroleum oils as insecticides. Plant Physiol. 4: 299–321. 1929.
7. Minshall, W. H. and Helson, V. A. The herbicidal action of oils. Proc. Amer. Soc. Hort. Sci. 53: 294–298. 1949.
8. Oberle, G. D., Pearce, G. W., Chapman, P. J., and Avens, A. W. Some physiological responses of deciduous fruit trees to petroleum oil sprays. Proc. Amer. Soc. Hort. Sci. 45: 119–130. 1944.
9. Ralston, A. W., Christensen, C. W., and Josh, G. Use of mercurated fatty compounds as weed killers. Oil & Soap 14: 5–7. 1937.
10. Scarth, G. W., Loewy, A., and Shaw, M. Use of the infrared absorption method for estimating the time course of photosynthesis and transpiration. Canadian Jour. Research C, 26: 94–107. 1948.
11. Wedding, R. T., Riehl, L. A., and Rhoads, W. A. Effect of petroleum oil spray on photosynthesis and respiration in citrus leaves. Plant Physiol. 27: 269–278. 1952.
12. Young, P. A. and Morris, H. E. Injury to apple by petroleum oil sprays. Jour. Agr. Research 47: 505–522. 1933.

THE ROLE OF BORON IN THE TRANSLOCATION OF ORGANIC COMPOUNDS IN PLANTS[1,2]

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The essentiality of boron for higher plants was demonstrated in 1910 (1). Since then several thousand reports have appeared and numerous physiological roles have been ascribed to boron. The literature has been reviewed by Gauch and Dugger (7) and interpreted in the light of some of the more recent evidence. They (6) recently postulated that boron may be necessary in order for sugar to move through the protoplasmic membranes. According to their theory, boron may react with the sugar molecule to form a complex which then moves through the membrane with greater facility than the sugar molecule itself. They also recognized that boron might instead be associated with the membrane, reacting with the sugar molecule at this locus. They believed that the evidence favors this latter mechanism.

Many authors have concluded that the high concentration of sugars in the leaves of boron-deficient plants was due to a breakdown of the conductive tissues with a concomitant reduction in translocation. Most of these studies have been conducted on plants

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showing morphological symptoms. There is some evidence (6, 12) that boron deficiency has an effect on the translocation of sugars, and possibly other organic compounds, before vascular derangement occurs.

In this study, an attempt was made to compare the translocation of carbohydrates in plants grown in a boron-sufficient nutrient solution with that of plants grown in a boron-deficient nutrient solution and to correlate this information with the observed symptoms of boron deficiency. Experiments were conducted on plants soon after the boron supply was removed, as well as on those showing symptoms of the deficiency.

Materials and Methods

Culture Methods: Marglobe tomato (*Lycopersicum esculentum* Mill.) plants were used in all of the experiments. It has been shown that the boron requirement for tomato is not altered by photoperiod (15). Lights were used, however, during the winter months to augment photosynthesis and thus to improve plant growth.

Seedlings were produced in flats containing sand. At the desired stage of growth, they were transplanted to automatically-irrigated sand culture equipment similar to that described by Gauch and Wadleigh (8). Five, 4, and 2 ml of molar solutions of $Ca(NO_3)_2 \cdot 4\,H_2O$, KH_2PO_4, and $MgSO_4 \cdot 7\,H_2O$, respectively, were added per liter of de-ionized water. Using monosodium, iron, ethylene-diaminotetraacetate (5000 ppm Fe) and a "supplementary solution" (50 ppm Zn as $ZnSO_4 \cdot 7\,H_2O$, 16 ppm Cu as $CuSO_4 \cdot 5\,H_2O$, and 500 ppm Mn as $MnCl_2$) 0.6 ml and 1 ml, respectively, of each of these two stock solutions were added to each liter of the nutrient solution. Boron, as H_3BO_3, was either absent or present in varied concentrations.

For the early growth of plants, 0.05 ppm of added boron appeared to approach sufficiency but, to ensure an adequacy, 0.1 ppm of boron was generally employed. Unless otherwise indicated, plants were grown to the desired stage with 0.1 ppm of boron. At this time the solutions were replaced with de-ionized water for 12 hours to free the cultures of boron. Following this treatment, the nutrient solutions were replenished, omitting boron in those solutions which were to be deficient. Usually solutions were changed weekly during the course of a given experiment.

Using the carmine method (9), attempts were made to determine the amount of boron in the de-ionized water but, even after concentrating 100-fold, none could be detected. Since a progressive increase in growth throughout the following portion of the range of boron concentrations, 0, 0.002, 0.005, 0.0075, 0.01, 0.015, and 0.02 ppm was observed, it would appear that the boron content of the de-ionized water must have been very low.

Experiments with C^{14}-labeled Sucrose: Experiments on the absorption and translocation of sugar in tomato plants were performed in the greenhouse. The third leaf from the tip of each plant was excised so as to leave intact as much of the petiole as possible. The tip of the petiole at this node was then immersed in 3 ml of a 7 % solution of uniformly-labeled sucrose having a specific activity of 0.0343 μc per ml. After the desired length of time, the plants were fractioned and dried in a hot-air, forced-draft oven at 70° C prior to grinding and the preparation of samples for counting.

Experiments with $C^{14}O_2$: Experiments involving CO_2 were performed in an atmosphere containing 0.5 % CO_2 so that the system would not be depleted of CO_2 during the course of the experiments. The total amount of C^{14} in the closed system was varied between 20 and 40 microcuries. The C^{14} was obtained as $BaCO_3$ and was converted to $NaCO_3$ in water to facilitate precision in the measurement of aliquots. Boron-deficient and sufficient plants were carefully potted from sand culture, watered, and placed in the dark for 24 hours to lower the internal carbohydrate level. One intact leaf of each plant was carefully placed in a desiccator (exposure chamber), with modeling clay being inserted in the notch in the lid to make a seal around the petiole.

After a predetermined amount of evacuation, $C^{14}O_2$ was released in a separate chamber (desiccator) with H_3PO_4 and circulated with a model T-4 Sigmamotor rubber-tubing pump for 10 minutes to allow the system to equilibrate. Light (approximately 250 fc) was supplied by two 200-watt incandescent light bulbs and a fluorescent light in the top of the hood. Lights were adjusted with the aid of a light meter so that the illumination would be as uniform as possible. The CO_2 was circulated within the closed system during the course of the experiment. Under these circumstances, preliminary experiments indicated that the temperature in the chamber did not rise more than 2° C above room temperature. After the desired length of time, plants were pressed and dried for radioautographs or fractioned and dried in an oven at 70° C.

Samples for radioassay were prepared on rings covered with celluloid tape, as described by Mitchell and Linder (13), and counted with a windowless flow counter. The weight of the samples was generally about 15 mg. Radioautographs were prepared in the usual manner with Kodak x-ray film. Exposure time was 2 days.

Chromatographic Techniques: After killing in boiling ethanol, the leaves were extracted in a Soxhlet, and the soluble materials were concentrated on a steam bath.

Organic acids were separated (3) with n-butyl alcohol, acetic acid, and water (4 : 1 : 1, v/v) and (5) ether, acetic acid, and water (13 : 3 : 1, v/v). Amino acids and sugars were separated with n-butyl alcohol, acetic acid, and water (4 : 1 : 1, v/v) and phenol and water (3 : 1, v/v). The indicators were bromcresol green for organic acids, ninhydrin for amino acids, naphthoresorcinol for sucrose, and benzidine for glucose and fructose. The general methods, outlined by

Block et al (3) for indicators and solvents, were employed.

Results and Discussion

Morphological Symptoms: Johnston and Dore (10) and Johnston and Fisher (11) have described boron-deficiency symptoms on tomato. There are numerous other reports of boron-deficiency symptoms in general. Many of the symptoms of boron-deficiency indicate a possible deficiency of carbohydrates. The fact that root tips die first (4) is indicative since they are close to the boron which is available but are far from the carbohydrate supply. During the seedling stage, the cotyledons of boron-deficient plants, grown in sand culture, enlarged to approximately twice the size of those of normal plants. This might indicate that boron-deficient plants are capable of expanding tissues near a source of supply of carbohydrate, but that they are not capable of developing new leaves to which large amounts of carbohydrates must be translocated. Young boron-deficient tomato plants, which have several sets of leaves, develop axillary shoots after the tips die (10). This again may be a response to an inadequacy of the mechanism for translocation. It is evident that an imbalance of hormone, associated with the death of the tip, could also be involved.

Applied Sugars: Several authors have implied that boron may be directly involved in the translocation of sugars (6, 12, 14). Inasmuch as stem-tips are among the first parts to die, attempts were made to prevent this occurrence by an application of aqueous sugar solutions to the tips. Solutions containing 4 % glucose, fructose, or sucrose were sprayed on the tips of plants that were receiving a boron-deficient solution. There was no apparent improvement of the "boron-deficient" plants receiving these applications of sugar. These results may have been caused by a failure of the applied sugars to penetrate even the outer layer of cells when the plants were boron deficient.

By immersing the tips of the petiole at the third node (leaf blade excised) in a 7 % C^{14}-labeled sucrose solution, the plants absorbed the sucrose and translocated it (or its hydrolytic products), so that a detectable amount was in the tips within two hours. Translocation was primarily to the tip and the first leaf below the tip, although the second leaf below was sometimes as active as the first leaf. Other leaves did not have a detectable amount of radioactivity in them. As shown in table I, plants grown in the absence of boron, but not yet showing visible symptoms of boron deficiency, translocated more sucrose (or its hydrolytic products) when 50 ppm of boron were added to the solution than when sucrose alone was applied. Normal plants, grown with 0.5 ppm boron, did not show a consistent increase in translocation when boron was added to the sugar solution.

When plants were showing morphological boron-deficiency symptoms, the uptake and translocation of applied sucrose to all parts of the plant were reduced (table II). The addition of boron at this stage apparently did not facilitate the translocation of sugar. Boron appears to aid in the translocation of carbohydrates before morphological symptoms are visible, but it does not increase the amount of translocation

Table I

Effect of Boron on the Distribution of Radioactivity in Normal and Boron-deficient Tomato Plants. C^{14}-Labeled Sucrose Was Introduced as a Solution through the Stump of the Petiole at the Third Node from the Tip. (Each Value from a Sample Composed of Duplicate Plants)

Plant part	Cpm/10 mgs of dry plant material			
	Normal plant		Deficient plant	
	Sucrose applied	Sucrose and boron applied	Sucrose applied	Sucrose and boron applied
	2-hour sampling			
Tip	18	10	9	30
First leaf	13	22	8	52
Second leaf	7	4	4	26
Average	13	12	7	36
	3-hour sampling			
Tip	18	27	23	35
First leaf	19	43	30	79
Second leaf	6	28	53	23
Average	14	33	35	46
	5-hour sampling			
Tip	52	28	35	72
First leaf	74	31	58	128
Second leaf	16	28	15	33
Average	47	29	36	78
	7-hour sampling			
Tip	60	164	70	87
First leaf	104	120	89	145
Second leaf	31	50	64	109
Average	68	111	74	114

Table II

Effect of Boron on the Distribution of Radioactivity in Normal and in Plants Showing Boron Deficiency Symptoms. C^{14}-Labeled Sucrose Was Introduced as a Solution through the Stump of the Petiole at the Third Node from the Tip. (Each Value from a Sample Composed of Duplicate Plants)

Plant part	Cpm/10 mgs of dry plant material			
	Normal plant		Deficient plant	
	Sucrose applied	Sucrose and boron applied	Sucrose applied	Sucrose and boron applied
Tip	100	100	18	35
Upper leaves	66	97	30	17
Lower leaves	36	38	10	1
Stem	64	55	16	30
Roots	49	51	14	15
Average	65	68	18	20

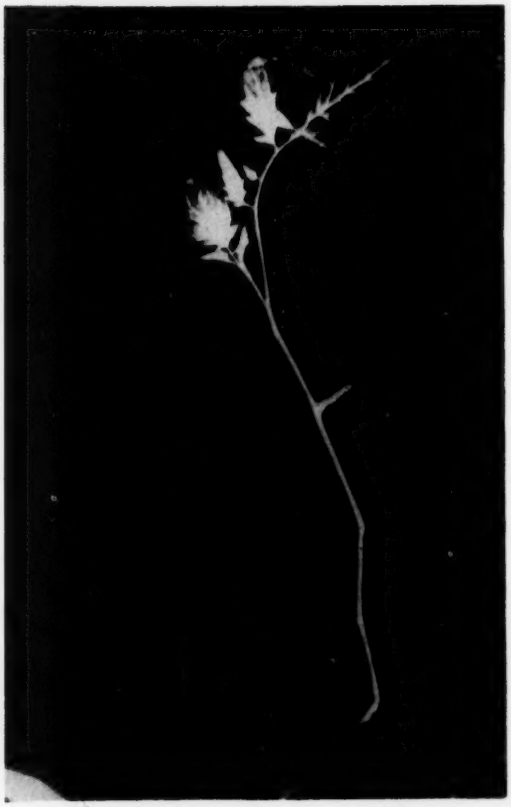

FIG. 1. Radioautograph illustrating the distribution of C^{14} in boron-sufficient plants after 4 hrs of photosynthesis. (Arrow indicates position of exposed leaf. Roots are light area at left, bottom.)

after symptoms are visible. The results of this experiment suggest that perhaps injury to the phloem had occurred which applied boron could not alleviate—at least during the course of these experiments.

$C^{14}O_2$ EXPERIMENTS WITH PLANTS SHOWING BORON-DEFICIENCY SYMPTOMS: Plants were subjected to $C^{14}O_2$ to determine whether the normal distribution pattern of the photosynthetic products would be altered when plants were boron deficient. Leaves of normal tomato plants and those of plants showing severe symptoms of boron deficiency were placed in a specially-designed chamber and exposed to 20 microcuries of $C^{14}O_2$ for 4 hours.

Radioautographs of these plants indicated that most of the photosynthetic products were translocated into the tip, first leaf below the tip, and the roots of normal plants (fig 1). Only traces of radioactivity were found in the other leaves. When plants were showing severe symptoms of boron deficiency, most of the translocation of the photosynthetic products was also to these same parts. The veinal pattern in normal plants was somewhat more distinct than that in boron-deficient plants. Those areas or regions of the plant usually associated with boron deficiency symptoms received the major portion of the photosynthate. This was true even when symptoms were severe (fig 2). The relative amounts of the photosynthate translocated to each region can be better evaluated from the counting data (table III).

The counting data showed that a greater percentage of the fixed C^{14} was translocated by boron-sufficient plants than by boron-deficient plants. This was more evident in the basal than in the apical portion. The magnitude of this difference could easily account for the death of plant parts or organs with inherently rapid rates of respiration. This would be especially true of the roots since they are farthest from the source of production of carbohydrates. It is possible that other essential compounds, which must be translocated from one part of the plant to another, would not be as readily translocated. Alexander (2) and Chandler (4) reported that boron-deficient plants lose their capacity to respond to gravity. This would indicate that there is some relation between boron and the production, translocation, or action of hormones. Mitchell et al (12) found that

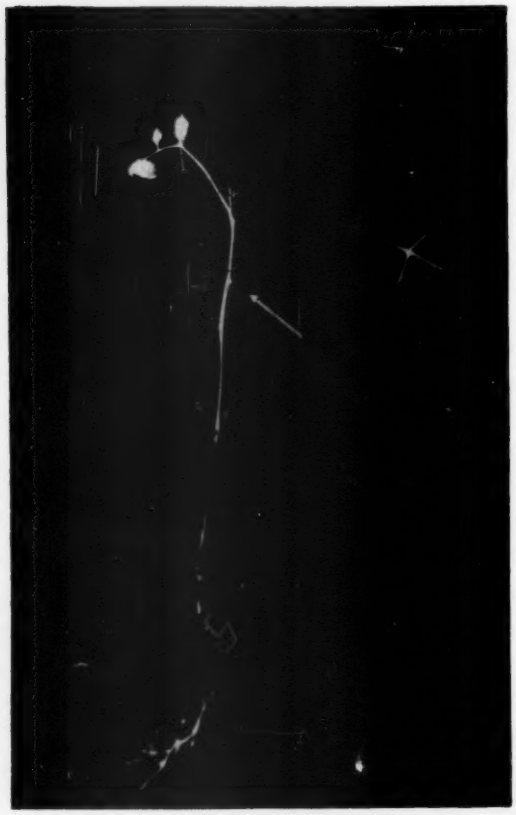

FIG. 2. Radioautograph illustrating the distribution of C^{14} in boron-deficient plants after 4 hrs of photosynthesis. (Arrow indicates position of exposed leaf.)

TABLE III

DISTRIBUTION OF THE PHOTOSYNTHETIC PRODUCTS OF BORON-DEFICIENT AND BORON-SUFFICIENT PLANTS AFTER 4 HRS OF PHOTOSYNTHESIS IN AN ATMOSPHERE CONTAINING $C^{14}O_2$. (EACH VALUE FROM A SAMPLE COMPOSED OF DUPLICATE PLANTS)

PLANT PART	C^{14} AS % OF TOTAL AMOUNT FIXED PER PLANT					
	EXPT I 9/18/54		EXPT II 9/21/54		EXPT III 9/24/54	
	BORON SUFFICIENT PLANT	BORON DEFICIENT PLANT	BORON SUFFICIENT PLANT	BORON DEFICIENT PLANT	BORON SUFFICIENT PLANT	BORON DEFICIENT PLANT
Tip	1.9	1.7	0.7	2.1	0.2	0.3
First leaf	...	...	...	...	0.7	0.2
Stem above treated leaf	5.2	2.2	8.3	6.2	11.0	7.5
Stem below treated leaf	4.3	6.5	19.7	3.2	7.0	1.6
Other leaves	0.3	0.5	1.8	7.5	0.7	0.1
Roots	5.9	1.6	1.3	0.8	0.2	0.4
Total	17.6	12.5	31.8	19.9	19.8	10.1

2,4-D and other growth-modifying substances were more rapidly translocated when boron and sugars were also applied. A decrease in the translocation of various compounds could be involved in the appearance of boron-deficiency symptoms but, as with hormones, the effect of boron on the translocation of carbohydrates may be the main effect.

$C^{14}O_2$ EXPERIMENTS WITH PLANTS NOT SHOWING BORON-DEFICIENCY SYMPTOMS: In order to determine how the rate of translocation was altered over short intervals of time when boron was absent from the nutrient solution and to determine at what stage of boron deficiency a difference in translocation could be noted, a series of plants was exposed to 20 μc of $C^{14}O_2$ for 20 minutes. At the completion of the exposure period the plants were fractioned, dried, and prepared for radioactivity assay. The plants were grown to about 10 inches in height with 0.1 ppm of boron, and then half of them were placed on a boron-free nutrient solution. The others were continued with 0.1 ppm of boron. Boron-deficient plants showed the characteristic symptom of brittleness at the end of 8 days and were just beginning to show other morphological symptoms at the end of 14 days when the experiments were discontinued.

Plants exposed to 20 μc of C^{14} for 20 minutes had a measurable amount of radioactivity in the first 4 cm up and down the stem from the point of attachment of the C^{14}-exposed leaf. After 4 days, plants lacking boron showed a decrease in the amount of fixed carbon that was translocated, as compared to normal plants treated in the same manner (table IV). With the exception of the eighth day, this decrease was observed throughout the experiment. On this day one of the duplicate boron-deficient plants was lost.

Since 20 μc of $C^{14}O_2$ did not result in a detectable amount of radioactivity beyond 4 cm up and down the stem, the experiment was repeated with 40 μc of $C^{14}O_2$, and 30 minutes (20 min of light; 10 min dark) were allowed for translocation. It was then possible to measure activity in the first 8 cm up and down the stem.

Vernon and Arnoff (16) found that the radioactivity in soybean plants treated with $C^{14}O_2$ for 20 minutes decreased logarithmically with the distance from the point of attachment of the treated leaf. This was also found to be the case in these experiments with

TABLE IV

PERCENT OF TOTAL PHOTOSYNTHATE TRANSLOCATED AFTER 20 MIN OF PHOTOSYNTHESIS WITH 20 MICROCURIES OF $C^{14}O_2$. (EACH VALUE FROM A SAMPLE COMPOSED OF DUPLICATE PLANTS)

DAYS ON TREATMENT	NO BORON ADDED	0.1 PPM OF BORON ADDED	% INCREASE DUE TO BORON
2	0.32	0.29	−12
4	0.42	0.78	81
6	0.55	1.63	214
8	0.17	0.16	− 6
10	0.08	0.15	88
14	0.05	0.07	40

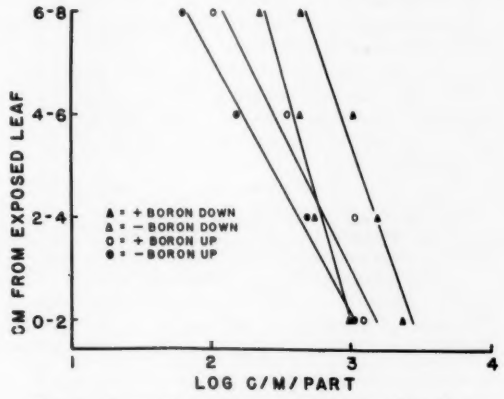

FIG. 3. Logarithmic plot of C^{14}-radioactivity in 2-cm stem segments of boron-deficient and boron-sufficient tomato plants treated with 40 μc of $C^{14}O_2$. In light, 20 min; darkness, 10 min.

tomato plants. Samples were counted in 2-cm segments to include a distance of 8 cm up and down the stem from the locus of the treated leaf. The activity was not sufficiently high in the sections of the stem beyond 8 cm to be determined accurately. The early stages of boron deficiency did not alter this logarithmic pattern of distribution. Movement in both boron-deficient and sufficient plants was usually greater in a downward than in an upward direction in the stem. A typical logarithmic plot of the activity found in these segments is shown in figure 3.

The total amount of C^{14} fixed by boron-deficient plants did not vary much from that of controls. The variation in the per cent of translocation from one interval to the next was largely due to unknown factors. It must be borne in mind, however, that treatment started at a stage of vigorous growth and that plants treated on the fourteenth day were about twice as large as those treated on the second day. The leaf selected for exposure on any given day was that leaf nearest the middle of the plant. As the plants developed, this resulted in a shift in the stage of development of the exposed leaf. Care was taken in selecting plants so that the boron-deficient and sufficient plants would have morphologically equivalent leaves on any given day.

In all cases boron-sufficient plants translocated a greater percentage of the C^{14} fixed, on any given day, than did comparable boron-deficient plants (table V). The amount of translocation in boron-deficient plants dropped to a low level after only 2 days and remained lower than that of boron-sufficient plants during the course of the experiment.

Chromatograms of Soluble Plant Material: Chromatograms made from leaf and stem extracts did not indicate large differences in organic acids between boron-deficient and sufficient plants. Citric, malic, succinic, and an unknown acid whose R_f corresponded to published values for α-ketoglutaric acid, were well resolved. Visual estimation of the amount of each acid indicated relatively little difference.

Chromatograms of amino acids resolved into eight spots. No visual difference was apparent between boron-deficient and boron-sufficient stems and leaves. Similarly little difference was apparent in the proportionalities of glucose, fructose, and sucrose in the leaves of boron-deficient and sufficient plants.

Table V

Percent of Total Photosnythate Translocated after 20 Min of Photosynthesis and 10 Min of Darkness with 40 Microcuries of $C^{14}O_2$. (Each Value from a Sample Composed of Duplicate Plants)

Days on Treatment	No Boron Added	0.1 Ppm of Boron Added	% Increase Due to Boron
2	0.24	2.41	900
4	0.14	0.28	100
6	2.21	2.98	35
8	0.77	1.60	108
10	0.24	0.64	167
14	1.18	1.66	49

Radioautographs of chromatograms of extracts of plants exposed to $C^{14}O_2$ for 20 min indicated that the majority of the photosynthate soluble in 80 % alcohol was represented by glucose, fructose, and sucrose in the exposed leaf in both boron-deficient and sufficient plants. Of the translocation products, extracted from boron-deficient plants, sucrose was by far the dominant constituent. The translocation products of boron-sufficient plants were not determined.

It is evident that the lack of boron decreased the amount of translocation to all parts of the plant soon after the boron supply was removed. Though the details of the action of boron in the translocation of carbohydrates are not yet clear, a mechanism similar to that proposed by Gauch and Dugger (6) would agree with the experimental evidence presented here.

Summary

A role of boron in the translocation of sugars was verified by these experiments with tomato plants in sand culture.

Sugar sprays applied to the tips of boron-deficient plants did not prevent death of the tips, but radioactive sucrose was more rapidly translocated in boron-deficient plants not yet showing morphological symptoms in the presence of 50 ppm of boron than in the absence of added boron. Once morphological symptoms of the deficiency were visible, sucrose uptake and translocation were reduced and applied boron did not aid in the distribution of the applied sugar.

After 4 hours of exposure to $C^{14}O_2$ a greater percentage of the photosynthate was translocated in boron-sufficient plants than in those showing boron deficiency symptoms. This was more apparent in the basal than in the apical portions of the plant.

After 20 min exposure to $C^{14}O_2$, distribution of C^{14} was found to decrease logarithmically with distance up and down the stem from the point of attachment of the exposed leaf. Boron-sufficient plants translocated a greater percentage of the photosynthate as early as 4 days after the boron supply was removed in one experiment and as early as 2 days in another experiment. With only one exception, this was found throughout the 14 days of experimentation.

Chromatographically there were no apparent differences in the concentrations of amino acids and organic acids in the two kinds of plants, and sugars were in the same proportions. Presumably sugar was moving primarily in the form of sucrose.

Literature Cited

1. Agulhon, H. Emploi du bore comme engrais catalytique. Compt. rend. acad. sci., France 150: 288–291. 1910.
2. Alexander, T. R. Anatomical and physiological responses of squash to various levels of boron supply. Bot. Gaz. 103: 475–491. 1942.
3. Block, R. J., LeStrange, R., and Zweig, R. Paper Chromatography. Pp. 1–195. Academic Press Inc., New York 1952.
4. Chandler, F. B. Mineral nutrition of the genus

Brassica with particular reference to boron. Ph.D. thesis, University of Maryland, College Park, Maryland 1939.
5. Denison, F. W., Jr. and Phares, E. F. Rapid method for paper chromatography of organic acids. Anal. Chem. 24: 1628. 1952.
6. Gauch, H. G. and Dugger, W. M., Jr. The role of boron in the translocation of sucrose. Plant Physiol. 28: 457–466. 1953.
7. Gauch, H. G. and Dugger, W. M., Jr. The physiological action of boron in higher plants: A review and interpretation. Agr. Expt. Sta., Maryland, Tech. Bull. A 80. 1954.
8. Gauch, H. G. and Wadleigh, C. H. A new type of intermittently irrigated sand culture equipment. Plant Physiol. 18: 543–547. 1943.
9. Hatcher, J. T. and Wilcox, L. V. Colorimetric determination of boron using carmine. Anal. Chem. 22: 567–569. 1950.
10. Johnston, E. S. and Dore, W. H. The influence of boron on the chemical composition and growth of the tomato fruit. Plant Physiol. 4: 31–62. 1929.
11. Johnston, E. S. and Fisher, P. L. The essential nature of boron to the growth and fruiting of the tomato fruit. Plant Physiol. 4: 387–392. 1930.
12. Mitchell, J. W., Dugger, W. M., Jr., and Gauch, H. G. Increased translocation of plant growth modifying substances due to application of boron. Science 118: 354–355. 1953.
13. Mitchell, J. W. and Linder, P. J. Some methods used in tracing radioactive growth-regulating substances in plants. Bot. Gaz. 112: 126–129. 1950.
14. Schropp, W. and Arenz, B. Über die Wirkung einiger spurenelemente, insbesondere des Bors, auf das Wachstum verschiedener Baumwollsorten. Phytopath. Zeits. 12: 366–404. 1939.
15. Struckmeyer, B. Esther and MacVicar, R. Further investigations on the relation of photoperiod to the boron requirement of plants. Bot. Gaz. 109: 237–249. 1948.
16. Vernon, L. P. and Arnoff, S. Metabolism of soybean leaves. IV. Translocation from soybean leaves. Arch. Biochem. Biophys. 36: 383–393. 1952.

NECESSITY OF INDOLEACETIC ACID FOR THE DUPLICATION OF CROWN-GALL TUMOR CELLS[1,2]

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The presence of a crown-gall tumor in vivo is frequently associated with numerous modifications of the growth behavior of the host. Included among these are severe epinasty, suppression of the development of lateral shoots, delay in petiolar abscission, and increased cambial activity. Many investigators found that indoleacetic acid (IAA) could induce almost identical abnormalities independent of the presence of a tumor (1, 14). It was postulated by Link and Eggers (18) that crown-gall tumors contained high concentrations of IAA that were carried throughout the host plant and were responsible for the observed changes. Indeed, bioassays of tomato plants demonstrated that there was much more auxin in tumor tissues than in corresponding normal stem tissues (18). This report could not at the time be confirmed (24), but the present evidence supports the findings of Link and Eggers. Although it was not then known whether the crown-gall bacteria synthesized the IAA present in tumor tissues or whether it was formed in the tumor cells, DeRopp (3) later concluded that the tumor cells themselves were the primary source of the auxin. Both Kulescha (15) and Henderson and Bonner (10) demonstrated that there was more IAA in the tissues of sterile tumors in vitro as compared with in vitro cultures of normal tissues and that IAA was synthesized by tumor cells.

Attempts to prove that these high endogenous levels of IAA are necessary for the duplication of tumor cells have failed since additions of IAA to crown-gall tissue in vivo or in vitro were generally inhibitory or without significant effect on tumor-cell duplication (3, 11, 15). The simplest way to demonstrate a positive role for IAA in the growth of crown-gall tissues would be to reduce the biologically effective level of IAA with concomitant reduction in growth. Control rates of growth should be restored by addition of this growth substance.

Two lines of attack on this problem have appeared. The first of these is the radiation technique discovered by Skoog (28) and developed by Gordon (6, 7, 35). Here, controlled doses of ionizing radiation reduce the endogenous level of IAA, presumably by blocking its synthesis. The doses of radiation used are not permanently inhibitory to other cellular processes, although higher doses have profound effects on chromosomes, nucleic acid biosynthesis, and other processes (22, 23) which are not reversible with IAA. Levin and Levine (19) and Rivera (25) found that hard x-rays would prevent the growth of tumors on Ricinus as judged by increases in tumor size or by microscopic examination of the affected cells. Stapp and Bortels (29), however, were unable to observe any reduction in tumor growth on tomato plants following x-irradiation. Recently, Waggoner and Dimond (33) demonstrated that ionizing radiations delayed the appearance of

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tumors in tomato and suppressed the growth of previously formed tumors. None of these workers attempted to reverse the radiation-induced inhibitions of tumor-cell growth with exogenous supplies of IAA.

The second technique now available for reducing the biologically effective level of IAA in tissue requires a specific antimetabolite. Experiments of this type, to be of positive value, must result in inhibitions of growth by the presumed antimetabolite which may be reversed by appropriate concentrations of the metabolite, in this instance, IAA. Interpretation of the results may be complicated by the fact that exogenous supplies of IAA may be inhibitory when supplied to tumor tissues which contain endogenous IAA. Thus, both antimetabolite and metabolite may be independently inhibitory, and inhibitions induced by either compound should be reversible by the other (5). The reports of McRae and Bonner (21) and Gentile and Klein (5) suggested that 2,4,6-trichlorophenoxyacetic acid (TCPA) might be a specific antimetabolite for IAA.

The experiments reported at this time, using both radiation and antimetabolite approaches, give presumptive evidence that IAA is, in fact, required for the duplication of the crown-gall tumor cells which synthesize it.

Materials and Methods

Radiation Studies: Tomato plants (var. Bonny Best) were grown from seed in flats of garden loam. Plants were selected for uniformity at the three leaf stage and repotted in glass shell vials of one inch diameter. After allowing several days for recovery from transplanting injuries, test plants were inoculated with *Agrobacterium tumefaciens* (strain S 5-6) by a single puncture through the second apical internode with a needle dipped into an 18-hour broth culture of the bacteria. Control plants received sterile punctures. All plants were kept on a greenhouse bench for 108 hours to permit complete transformation of normal cells into primary tumor cells (14). They were then irradiated with x-rays, gamma-rays, or fast neutrons at the dose levels given in table I. X-rays were supplied by a General Electric Maximar x-ray unit at 250 KV and 15 milliampere with a 1-mm copper filter. Dose rate was controlled by altering the distance between the source and the zone of puncture. Exposures to the other ionizing radiations were carried out in a gamma-neutron radiation chamber (31) at Argonne National Laboratory's CP-3 reactor. Fast neutrons were produced through fission, by allowing slow neutrons from the thermal column of the reactor to impinge upon a uranium plate. Appropriate shielding devices of boron carbide prevented slow neutrons from reaching the plants. The gamma contamination of the fission neutrons was approximately 7 % of the total physical dose. Pure gamma radiation was obtained from 18 sources of cobalt^{60} at the opposite end of the chamber from the uranium converter plate.

Neutron dose was measured in roentgen equivalents physical (rep) with Victoreen condenser r-chambers which had been calibrated against a tissue equivalent chamber of 10.1 % hydrogen composition (27) and a Hurst-Ritchie proportional counter (12). For gamma rays, the same condenser chambers were calibrated by the U. S. National Bureau of Standards.

Table I

Inhibition of the Duplication of Crown-gall Tumor Cells in Vivo by Ionizing Radiation and Reversal of Inhibition with Indoleacetic Acid *

Radiation	Dose r or rep/min	Total dose	Av. tumor diameter as % of control ***	
			Lanolin	Lanolin ** IAA
Fast neutrons	3.4	340 rep	95	103
	3.4	550 rep	28	100
	4.6	950 rep	7	90
X-rays	10.6	500 r	99	100
	10.2	1000 r	76	98
	12.2	1500 r	8	80
	47.5	5000 r	7	7
Gamma rays	12.2	1500 r	106	106
	12.5	2100 r	58	101
	12.2	2850 r	7	90

* Tomato stems inoculated 108 hr prior to irradiation.

** Lanolin pastes applied to surface of bisected internode bearing this tumor.

*** Tumor diameter measured 18 days after irradiation and treatment. Control = tumors on untreated plants.

The plants were irradiated in a single vertical plane in a Lucite exposure box, 16 inches high, 16 inches wide, and two inches thick. Approximately 30 plants were irradiated simultaneously, half the vials arranged in the bottom of the box, the other half placed in a shelf eight inches above. The intensity of the radiation field was carefully mapped for all exposures with a Victoreen rate-meter. Total doses received at the zone of puncture on the stems of the tomato plants varied $\pm$ 5 % from the values listed in table I. Eight to 10 plants were used for each variable in all experiments.

Within one hour after irradiation, all plants were decapitated just below the apical node, leaving intact the second internode bearing the inoculation site. Lanolin or lanolin-IAA pastes (1 % IAA) were placed on the cut surfaces with a warmed syringe; each plant received 0.01 ml. The plants were transferred to pots of loam and kept on a greenhouse bench for 18 days. Axillary shoots formed during this period were removed to minimize growth responses to any other source of auxin. Tumor-cell duplication was judged by determination of the diameters of stem and tumor with a micrometer gauge. During the period of study, increases in tumor size was due almost entirely to the duplication of tumor cells (14).

Tissue cultures of a secondary crown-gall tumor of sunflower (36) were grown on White's medium for 20 days at 24 $\pm$ 2° C. The tissues were irradiated with

x-rays in the culture tubes, were weighed, transferred to fresh tubes of White's medium or White's medium containing 5 mg/l IAA, and were incubated for 30 days at 24° C. Final fresh weight values were determined and the "percentage growth" was calculated (final weight − initial weight/initial weight). Increase in fresh weight is here used as a measure of the growth by cell division since cell enlargement occurs infrequently under the conditions used (14).

ANTIMETABOLITE STUDIES: Sunflower tissue cultures were grown on White's medium; all media used for normal tissues was supplemented with naphthaleneacetic acid (NAA) at 10^{-7} gm/l. The following tissues were used: normal, habituated (9), secondary petiolar crown-gall (36), and primary stem crown-gall (2). In each case, several morphological types of tissue were noted in the cultures as received in this laboratory and, before being used, they were selected to consist of a single type. Indoleacetic acid and TCPA were each added to White's medium to final concentrations of 1 to 50×10^{-5} and 0.1 to 10×10^{-5} M, respectively. Mixtures of these compounds were made in all combinations with the concentrations used. Fragments of tissue were grown on control media for two weeks before being weighed to within 2 mg and were transferred to test media where they grew for 30 days at 27° C. Percentage growth values were determined and the data are reported as percentages of the percentage growth of tissue grown without supplements. Five replicates of each variable were set up; the standard deviation approximated 12 %.

RESULTS

RADIATION STUDIES: Fast neutrons were the most effective in suppressing tumor-cell duplication, 550 roentgen equivalents physical (rep) suppressing about 75 % of tumor growth as compared with non-irradiated control (table I). X-rays and gamma-rays were less effective than fast neutrons. Complete inhibition of tumor growth was achieved with 950 rep of fast neutrons, 1500 roentgens (r) of x-irradiation, or 2850 r of gamma-irradiation. With these doses, the addition of IAA reversed the inhibitions although 1500 r of x-irradiation affected metabolic activities not reversible with IAA since complete restoration of tumor growth did not occur. It is of interest that following 5000 r x-irradiation, the inhibition of tumor growth was not at all reversible with IAA. In contrast to the findings of Waggoner and Dimond (33), no spontaneous recrudescence of tumor growth was observed following any radiation treatment. It is noteworthy that suppression of growth was achieved with dose levels considerably below those used by Waggoner and Dimond.

Suppression of tumor-cell duplication by ionizing radiation and restoration of growth by IAA was also demonstrated with tissue cultures of crown-gall origin (table II). Five mg/l IAA (5.7×10^{-5} M) in the medium independently reduced growth to its half maximum level, essentially the same growth inhibition reported by Hildebrandt and Riker (11). X-irradiation to 1000 r total dose independently suppressed growth 68 %. When irradiated tissues were grown on media containing that level of IAA which reduced growth to half the control value, the growth was significantly superior to that obtained following irradiation alone or growth in the presence of IAA. Here, as with tumor tissues in vivo, IAA was capable of reversing the inhibitory effects of radiation and IAA appears to be necessary for growth of the tumor. On the basis of these experiments, and the results of Weber and Gordon (35), it appears that IAA is required for the duplication of tumor cells when they are rendered deficient in auxin by a radiation-induced blocking of IAA synthesis. These data indicate that IAA is required for the duplication of crown-gall tumor cells.

TABLE II

EFFECT OF IONIZING RADIATION AND INDOLEACETIC ACID ON GROWTH OF TISSUE CULTURES OF SECONDARY CROWN-GALL

TREATMENT	NO. TISSUES	MG FRESH WT		% GROWTH	STD DEVIATION	% CONTROL
		INITIAL	FINAL			
Control	10	242	569	130	16 %	100
Control + IAA *	10	267	442	66	13 %	51
Irradiated ** ...	15	263	372	42	18 %	32
Irradiated + IAA	15	265	545	106	14 %	82

* IAA = 5 mg/l.
** 1000 r total dose of x-rays given 10 r/min.

ANTIMETABOLITE STUDIES: Since this hypothesis would be strengthened if confirmed by an independent method, we turned to the antimetabolite technique for reducing the biologically effective level of IAA in the cells. The study was enlarged to include normal tissue cultures known to require an exogenous supply of IAA, or its equivalent, for growth, and habituated tissue which is presumed to be autotrophic for IAA. The sunflower tissues used differed in their growth rates; the 30-day increase in weight for normal tissues was 200 %; habituated tissues, 250 %; primary crown-gall tissue, 190 %; and secondary crown-gall tissue, 100 %. Thus, crown-gall tumor tissues do not necessarily grow at faster rates than do corresponding normal tissues.

For all tested tissues, both IAA and TCPA were independently inhibitory. To be considered as significant, stimulation, inhibition, or reversal of growth percentages of any variable must deviate from the appropriate control by at least 15 to 20 %. Table III gives the concentration of IAA and of TCPA necessary to reduce the percentage growth to 50 % of the control value. These data suggest that secondary petiolar crown-gall tissue was the least able to adjust to changes in the biologically effective concentration of IAA on either side of its endogenous level (which may be presumed to be near optimal). Primary tumor tissue was the most adaptable. Normal tissues

TABLE III

CONCENTRATIONS OF INDOLEACETIC ACID OF TRICHLOROPHENOXYACETIC ACID NECESSARY TO REDUCE THE GROWTH OF SUNFLOWER TISSUE CULTURES TO THE HALF MAXIMAL LEVEL

SUNFLOWER TISSUE CULTURE	IAA ($M \times 10^{-5}$)	TCPA ($M \times 10^{-5}$)
Normal	5.0	0.13
Habituated	1.0	1.0
Primary tumor	30.0	4.0
Secondary tumor	4.0	0.05

of sunflower, as might be expected, apparently can tolerate increased concentrations of IAA better than they can adjust to any reduction in IAA levels. Habituated tissues had a relatively narrow zone of tolerance.

Growth of the tested sunflower tissue in the presence of different combinations of IAA and TCPA showed a number of interesting correlations. For normal tissue (table IV), 1 and 5×10^{-5} M IAA significantly reversed the inhibitions of growth induced by 0.1×10^{-5} M TCPA and all tested concentrations of IAA reversed the inhibitions caused by 0.5×10^{-5} M TCPA. In one instance, the reversal of the inhibitions of each compound alone (double inhibition) was complete. TCPA at 1.0×10^{-5} M almost completely prevented the growth of the tissue. For normal tissues of sunflower, IAA and TCPA acted as an antimetabolite-metabolite system and IAA apparently is required for growth. This was expected since auxin must be supplied to this tissue for any growth.

For both primary and secondary crown-gall tissues which do not require an exogenous supply of auxin, similar patterns of inhibition and reversal were obtained. These data, too, are presumptive evidence that IAA is required for the duplication of these two types of crown gall tumor tissue.

Although both IAA and TCPA were independently inhibitory for habituated tissue, neither substance significantly reversed the inhibitions caused by the other. There was, however, evidence that they did interact since the addition of 50×10^{-5} M IAA to tissues grown with TCPA resulted in synergeistic inhibitions. Habituated tissue appears to differ qualitatively from normal and tumor tissues from the same species.

DISCUSSION

There is little doubt that IAA or its biological equivalent is required for the growth of most plant tissues. The previously noted failure of workers to demonstrate its necessity for crown-gall tumor tissues is now clear. Most tumor tissues possess the ability to maintain endogenously a level of IAA necessary for optimal growth. When an additional amount of IAA is supplied to them, the total level becomes supra-optimal and inhibition or "toxicity" is observed. Mature tissues in vivo and tissue cultures of normal cells, which usually do not synthesize sufficient IAA for optimal growth, may be stimulated by the addition of IAA in proper amounts. This is not to imply that the auxin-utilizing systems of crown-gall cells are qualitatively unique; they probably are quantitatively different from the cells from which they are derived. Although it would simplify the situation were high endogenous levels of IAA solely responsible for the observed growth behavior and metabolic patterns characteristic of crown-gall cells (14), this does not appear to be true. Many of the biochemical activities and morphogenic characteristics of tumor cells are quite different from normal cells or from neoplastic cells activated by auxins. One must conclude that the ability of crown-gall tumor cells to synthesize and to use the high levels of IAA found in them are only two of the activities which differentiate them from normal cells.

Examination of the data obtained by two techniques indicates that IAA is required for the multiplication of crown-gall tumor cells. Previous work showed that auxin is one of the agents necessary for the complete transformation of normal cells into primary tumor cells (14). Other work (30) suggests that IAA may be involved in the differentiation of mature tumor cells. It would appear that one substance—IAA—plays three major etiological roles in the life history of a crown-gall tumor cell.

It might be expected that anti-auxins would sup-

TABLE IV

INHIBITION OF THE GROWTH OF SUNFLOWER TISSUES IN VITRO BY EITHER INDOLEACETIC ACID OR 2,4,6-TRICHLOROPHENOXYACETIC ACID AND REVERSAL OF THESE INHIBITIONS BY THE OTHER COMPOUND. RESULTS EXPRESSED AS PERCENT OF THE PERCENTAGE GROWTH OF CONTROL CULTURES

TCPA ($M \times 10^{-5}$)	IAA ($M \times 10^{-5}$)					
	0.0	0.1	1.0	5.0	10.0	50.0
	Sunflower—Normal					
0.0	100	87	72	49	24	..
0.1	44	59	69	100 *	..	..
0.5	36	64	69	77 *	..	..
1.0	23	0	0	0	0	..
	Sunflower—Habituated					
0.0	100	..	58	..	55	34
0.1	96	..	86	..	68	16
1.0	49	..	39	..	52	18
5.0	25	..	22	..	19	8
10.0	20	..	11	..	17	12
	Sunflower—Primary crown gall					
0.0	100	..	113	..	74	39
1.0	75	..	78	..	90 *	29
5.0	40	..	52	..	25	17
10.0	28	..	47	..	22	11
	Sunflower—Secondary crown gall					
0.0	100	..	69	..	26	11
1.0	24	..	57	..	93 *	30
5.0	8	..	22	..	31	80 *
10.0	1	..	3	..	10	80 *

* Variables showing significant reversals of the double inhibitions.

press tumor growth in vivo. Roberts (26) reported that a naturally-occurring "anti-auxin" will reduce tumor growth on tomato plants and would also decrease the severity of the accompanying epinasty and adventitious root formation characteristic of the response of this host to IAA diffusing from the tumor. Waggoner and Dimond (34) reported that maleic hydrazide, a reputed anti-auxin, acted in a similar fashion but Klein and Klein (13) and Manil and Straszewska (20) had previously obtained negative results with this compound. The failure of maleic hydrazide to inhibit tumor-cell multiplication in vivo is in contrast to its effects in vitro where it severely reduced tumor growth (16), but it remains to be determined whether maleic hydrazide is an anti-auxin. The data presented here does show that TCPA can be used as an anti-auxin in vitro.

It is of interest that the data on TCPA reported here and those reported by Gentile and Klein (5) on Diplodia do not fit the kinetic theory developed by McRae and Bonner (21). The latter authors based their theory on the TCPA-IAA interactions on growth by cell elongation. Both Gentile and Klein and the present authors measured growth by increases in the weight of cell masses. In the Diplodia studies, as well as in the tissue cultures, growth is primarily by cell division. These differences in technique and results may indicate that IAA plays different roles in cell enlargement and in cell division (4).

The radiation data reported here are of interest independent of their relation to the IAA problem. Fission neutrons and gamma rays dissipate their energy in matter in distinctly different ways. Ions from gamma irradiation are more uniformly scattered throughout tissue, whereas fission neutrons, through proton recoil, cause more concentrated aggregates of ion pairs (17). This physical difference in specific ionization and linear energy transfer appears to be of significance in the relative biological effectiveness of these radiations. Although the data presented in table I are insufficient to justify setting a definite figure for the relative biological effectiveness, it is clear that fission neutrons are somewhat more effective and cobalt^{60} gamma rays less effective than 250 KVP x-rays for this biological criterion. These results confirm similar experiments in which a variety of organisms (mice, chicks, fruit flies, grasshoppers, kidney bean seeds, etc.) have been exposed in a gamma-neutron chamber (32).

Summary

The reduction of the endogenous or biologically effective level of IAA in crown-gall tumor tissues was accomplished in vivo and in vitro by the use of ionizing radiations (x-rays, gamma rays, and fast neutrons) and by the use of a specific anti-auxin. In all cases, the primary effects were a reduction in the growth of the tissues. These inhibitions of growth could be reversed wholly or in part by supplying the tumor cells with appropriate concentrations of IAA. These results give presumptive evidence that IAA may be specifically required for the duplication of crown-gall tumor cells which presumably synthesize optimal amounts of auxin.

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Literature Cited

1. Appler, H. Über die Tumorbildung durch *Pseudomonas tumefacians* und durch Heteroauxin an *Helianthus annuus*. Biol. Zentr. 70: 452–469. 1951.
2. DeRopp, R. S. The isolation and behaviour of bacteria-free crown-gall tissue from primary galls of *Helianthus annuus*. Phytopathology 37: 201–206. 1946.
3. DeRopp, R. S. The growth-promoting and tumefacient factors of bacteria-free crown-gall tissues. Amer. Jour. Bot. 34: 248–261. 1947.
4. DeRopp, R. S. and Markley, Elizabeth. The correlation of different aspects of auxin action. Plant Physiol. 30: 210–214. 1955.
5. Gentile, A. C. and Klein, R. M. The apparent necessity of indoleacetic acid for the growth of Diplodia (Fungi Imperfecti). Physiol. Plantarum 8: 291–298. 1955.
6. Gordon, S. A. Occurrence, formation, and inactivation of auxins. Ann. Rev. Plant Physiol. 5: 341–378. 1954.
7. Gordon, S. A. and Weber, R. P. The effects of x-irradiation on indoleacetic acid and auxin levels of the plant. Abstr. Amer. Jour. Bot. 37: 678. 1950.
8. Gordon, S. A. and Weber, R. P. Studies on the mechanism of phytohormone damage by ionizing radiation. Plant Physiol. 30: 200–210. 1955.
9. Henderson, J. H. M. The changing nutritional pattern from normal to habituated sunflower callus tissue in vitro. Anneé Biologique 3^{e} Ser. 30: 329–348. 1954.
10. Henderson, J. H. M. and Bonner, J. Auxin metabolism in normal and crown-gall tissues of sunflower. Amer. Jour. Bot. 39: 444–451. 1952.
11. Hildebrandt, A. C. and Riker, A. J. Influence of some growth regulating substances on sunflower and tobacco tissues in vitro. Amer. Jour. Bot. 34: 421–427. 1947.
12. Hurst, G. S. and Ritchie, R. H. Fast neutron dosimetry. Radiology 60: 464–468. 1953.
13. Klein, R. M. and Klein, Deana T. Effects of maleic hydrazide on initiation and development of tomato crown-gall tumors. Amer. Jour. Bot. 39: 727–730. 1952.
14. Klein, R. M. and Link, G. K. K. The etiology of crown-gall. Quart. Rev. Biol. 30: 207–277. 1955.
15. Kulescha, Zöia. Recherches sur l'elaboration de substances de croissance par le tissus végétaux. Thesis, Univ. Paris, Librarie General de l'Enseignment. 1951.
16. Kulescha, Zöia. Action de l'hydrazide maléique sur le proliferation des tissus de crown-gall de

Scorsonère et sur leur tenuer en auxine. Compt. rend. acad. sci., France 236: 958–959. 1953.
17. Lea, D. E. Actions of Radiations on Living Cells. Chapter 1, Pp. 1–32. The University Press, Cambridge. 1947.
18. Link, G. K. K. and Eggers, Virginia. Hyperauxiny in crown-gall of tomato. Bot. Gaz. 103: 87–106. 1941.
19. Levin, I. and Levine, M. The influence of x-rays on the development of crown-gall. Proc. Soc. Exptl. Biol. Med. 15: 24–25. 1917.
20. Manil, P. and Straszewska, Zofia. Action de l'hydrazide maléique sur les tumeurs du crown-gall. Bull. inst. agron. sta. recherches Gembloux 20: 128–131. 1952.
21. McRae, D. H. and Bonner, J. Chemical structure and anti-auxin activity. Physiol. Plantarum 6: 485–510. 1953.
22. Mika, E. S. Effect of indoleacetic acid on root growth of x-irradiated peas. Bot. Gaz. 113: 285–292. 1952.
23. Rasch, Ellen M. Nuclear and cell division in *Allium cepa* as influenced by slow neutrons and x-rays. Bot. Gaz. 112: 331–384. 1951.
24. Riker, A. J., Henry, B., and Duggar, B. M. Growth substance in crown-gall as related to time after inoculation, critical temperature and diffusion. Jour. Agr. Research 63: 395–405. 1941.
25. Rivera, V. Y. Depressione ed esalt azione dell' accrescemente in neoplasma vegetale sperimentale radiata. Riv. biol. 9: 62–69. 1927.
26. Roberts, R. H. A naturally occurring anti-auxin. Science 117: 456–457. 1953.
27. Rossi, H. H. The n-unit and energy absorption in tissue. Radiology 61: 93–96. 1953.
28. Skoog, F. The effects of x-irradiation on auxin and plant growth. Jour. Cellular Comp. Physiol. 7: 227–270. 1937.
29. Stapp, C. and Bortels, H. Der Pflanzenkrebs und seine Erreger, *Pseudomonas tumefaciens*. III. Zur frage der Bekämpfung. Zeits. Bakt. (II) 88: 313–317. 1933.
30. Struckmeyer, B. Esther, Hildebrandt, A. C., and Riker, A. J. Histological effects of growth regulating substances on sunflower tissue of crown-gall origin grown in vitro. Amer. Jour. Bot. 36: 491–495. 1949.
31. Vogel, H. H. Jr., Blomgren, R. A., and Bohlin, N. J. G. Gamma-neutron radiation chamber for radiobiological studies. Nucleonics 11: 28–31. 1953.
32. Vogel, H. H. Jr., Clark, J. W., and Jordan, D. L. The relative biological effectiveness of fast neutrons and Co^{60} gamma rays. Radiation Research 1: 233. 1954.
33. Waggoner, P. E. and Dimond, A. E. Crown-gall suppression by ionizing radiation. Amer. Jour. Bot. 39: 679–684. 1952.
34. Waggoner, P. E. and Dimond, A. E. Crown-gall suppression by anti-auxin. Science 117: 13. 1953.
35. Weber, R. P. and Gordon, S. A. Enzymatic radiosensitivity in auxin biosynthesis. Report A.I.B.S. meeting, Madison, Wisconsin, 1953.
36. White, P. R. and Braun, A. C. A cancerous neoplasm of plants. Autonomous bacteria-free crown-gall tissue. Cancer Research 2: 597–617. 1942.

EFFECT OF MANGANESE AND CERTAIN OTHER METAL CATIONS ON ISOCITRIC DEHYDROGENASE AND MALIC ENZYME ACTIVITIES IN PHASEOLUS VULGARIS[1,2]

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It is well established that many of the enzymes in plants require metal ions for activity. An examination of the effect of variable levels of mineral ions in culture media on enzyme activities of tissues offers possibilities of explaining the roles and interactions of metals in metabolism. Brown and Steinberg (2) have reported that leaves from tobacco plants grown in Fe-deficient media were low in peroxidase activity and that leaves from Cu-deficient plants were characteristically low in ascorbic acid oxidase activity. Another example of reduced activity of a metalloenzyme caused by a metal deficiency is the observation of Nicholas, Nason and McElroy (13) that nitrate reductase of Neurospora was markedly reduced when the fungus was grown in media containing insufficient Mo. Other metal deficiencies had little effect on nitrate reductase activity.

Decreased metalloenzyme activities of tissues of plants grown in media deficient in the metal associated with the enzyme are expected. The influence of a metal deficiency on the activity of enzymes not known to be directly associated with the particular metal in question is more difficult to interpret. The findings by Nason, Kaplan, and Oldewurtel (12) that Zn deficiency in Neurospora resulted in a striking increase in diphosphopyridine nucleotidase is of this type. It has also been shown (11) that Cu-deficient tomato leaves exhibited abnormally high isocitric dehydrogenase activity. This was interpreted in terms of a specific effect of Cu deficiency on protein synthesis and not on the basis of any direct relationship between Cu and the enzyme.

It has been established in numerous cases that high concentrations of certain metals apparently cause induced deficiencies of other metal cations. An example of such an interrelationship was shown by the work of

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Somers and Shive (16). Excessive concentrations of Mn added to culture solutions of soybeans resulted in symptoms that could be partially reversed by additional Fe, and excessive amounts of applied Fe resulted in symptoms that could be alleviated by additional Mn. Recently Weinstein and Robbins (18) reported that catalase and cytochrome oxidase activities of sunflower leaves were low under the conditions of either Fe deficiency or Mn toxicity. It also has been shown by Healy, Cheng, and McElroy (8) that toxic concentrations of Co in Neurospora cultures resulted in enzyme patterns similar to those of Fe deficiency. It seems that much more information is needed on the effect of metal levels in culture media and ratios of various metal ions in culture solutions on enzyme patterns of tissues.

It is the purpose of this communication to report the influence of variable cultural levels of Mn in relation to concentrations of certain other metal ions in solutions on isocitric dehydrogenase and "malic" enzyme activities of tissues. It has been reported that both of these enzymatic reactions are strongly activated by Mn^{++} and Co^{++} ions and to a lesser degree by Mg^{++} ions (4, 5). It is hoped to obtain a better understanding of the role of Mn in plant metabolism and of the factors that influence the utilization of this element.

Materials and Methods

Snapbean plants (*Phaseolus vulgaris*, var. Tendergreen) used in the various experiments were transferred at the seedling stage to aerated culture solutions in the greenhouse. The normal culture solution contained the following molar concentrations of major nutrient salts: 0.005 $Ca(NO_3)_2$, 0.002 $MgSO_4$, 0.002 K_2SO_4, 0.0005 K_2HPO_4 (adjusted to pH 5.5 with HCl). The concentrations of micronutrient elements in ppm were: 0.5 Fe as $FeSO_4$, 0.25 Mn as $MnCl_2$, 0.01 Zn as $ZnSO_4$, 0.01 Cu as $CuSO_4$, 0.25 B as $Na_2B_2O_7$, 0.015 Mo as Na_2MoO_4. In those experiments involving Mn deficiency the micronutrient salts were purified by the method of Stout and Arnon (17). Experiments were designed as randomized blocks with 3 replications. All treatments except metal toxicities were applied when seedlings were transferred into the culture solutions. Plants receiving metal toxicity treatments were grown in normal culture solutions until the appearance of the second set of trifoliate leaves (9 to 12 days). At that time the metal toxicity treatments were applied.

Cell-free extracts of leaves assayed in the various experiments were prepared by grinding with a cold mortar and pestle one weight of the second set of trifoliate leaves with 7 weights of 0.05 M tris-(hydroxymethyl)-aminomethane (TRIS) buffer at pH 7.4. The macerated tissue was transferred to a Ten Brock homogenizer, ground for 5 minutes, centrifuged at 20,000 × g at 0 to 2° C for 10 minutes, and the supernatant collected. Root extracts were prepared in a similar manner by grinding 2 weights of water-washed roots with 7 weights of cold TRIS buffer.

For the analysis of the type of metal inhibition of enzyme activities a 35 to 65 % $(NH_4)_2SO_4$ fraction of crude extract of leaves was collected by centrifugation and dissolved in 30 % volume (reference to crude extract) of 0.05 M TRIS buffer at pH 7.4. Before use it was dialyzed for 3 hours against 3 l of 0.01 M TRIS buffer at pH 7.4, and then for 2 hours against 3 l of a cold resin-water suspension prepared by mixing 10 gm of hydrogen saturated IR 120 with sufficient hydroxyl saturated IRA 400 (Rohm and Haas Company) until the supernatant liquid was at pH 6.5. After this treatment the enzyme activities without Mn^{++} were less than 5 % of that of the standard assay mixture which included this ion.

Isocitric dehydrogenase activity was determined by measuring the rate of reduction of triphosphopyridine nucleotide (TPN^+, Sigma Chemical Company, St. Louis) in a Beckman spectrophotometer at 340 mμ with a 1-cm light path. The reaction mixture contained the following materials expressed as micromoles: 300 TRIS buffer at pH 7.4, 9 D,L-isocitrate at pH 7.4, 6 $MnSO_4$, 0.2 TPN^+. The final volume of the reaction mixture was adjusted to 3 ml with water and the reaction started by the adition of 0.1 ml of extract. One unit of isocitric dehydrogenase was defined as that amount of enzyme which would cause an optical density change of 0.001 per minute. "Malic" enzyme activity was determined by a similar procedure with the exception that 9 micromoles of recrystalized L-malate at pH 7.4 were added instead of isocitrate. An optical density change of 0.001 per minute in this assay was defined as one unit of "malic" enzyme. Enzyme activities were expressed per mg of protein which was determined by the method of Lowry et al (10) using casein as the standard. The average protein content of leaf extracts was 6 mg per ml and that of roots 2 mg per ml.

It was necessary to determine whether or not the results of the "malic" enzyme assays were influenced by differences in lactic dehydrogenase activities of tissues. Reduced triphosphopyridine nucleotide (TPNH) for this assay was prepared by a reaction mixture containing the following materials expressed as micromoles: 600 TRIS buffer at pH 7.4, 100 $MgCl_2$, 7 glucose-6-phosphate, 5 TPN^+. The reaction was started by the addition of a solution containing 4 mg of crude glucose-6-phosphate dehydrogenase (Sigma Chemical Company, St. Louis) and the final volume adjusted to 6 ml with water. After cessation of reaction as dedetermined by maximum optical density at 340 mμ, the mixture was adjusted to a pH of 9.3, boiled for 3 minutes, and centrifuged. The assay mixture for the determination of lactic dehydrogenase contained the following materials expressed as micromoles: 300 TRIS buffer at pH 7.4, 10 pyruvate at pH 7.4, 6 $MnSO_4$, 0.025 TPNH. The reaction in a final volume of 3 ml was started by the addition of 0.1 ml of extract. The amount of disappearance of TPNH without pyruvate was subtracted from those containing pyruvate in determining lactic dehydrogenase activity. The amount of TPNH added in the lactic dehydro-

genase assays was approximately 3-fold greater than the amount of TPNH produced in the "malic" enzyme assays at the end of one minute. Tests for lactic dehydrogenase were also made by using reduced diphosphopyridine nucleotide (DPNH, Sigma Chemical Company, St. Louis) as the hydrogen donor.

Results

MANGANESE CULTURE TREATMENT AND ENZYME ACTIVITIES: Numerous experiments were carried out to test the effect of variable Mn levels in culture solutions on isocitric dehydrogenase and "malic" enzyme activities. Samples of tissues from plants of a typical experiment (table I) were assayed 4 to 6 days after the toxic concentration of Mn had been applied to the culture solutions. At this time plants grown without added Mn showed severe Mn-deficiency symptoms. The roots of plants from cultures receiving 10 ppm of Mn were dark brown and the leaves showed a chlorosis ordinarily associated with excessive quantities of this element.

When extracts prepared from Mn-deficient or normal plants were assayed without the addition of Mn^{++} to the reaction mixture, relatively little isocitric dehydrogenase or "malic" enzyme activity was observed. Similarly, Kraemer et al (7) have reported that certain $(NH_4)_2SO_4$ fractions of wheat germ exhibited no "malic" enzyme or isocitric dehydrogenase activity unless Mn^{++} was added during assaying. Both enzymes of extracts of Mn-toxic plants were highly active without the addition of Mn^{++} to the assay medium. In fact, the activities of the two enzymes from the roots of Mn-toxic plants were not further stimulated by additional Mn^{++}. This was true even though the fresh tissue had been diluted over 100-fold during preparation and assay.

TABLE I

EFFECT OF MANGANESE LEVEL IN CULTURE SOLUTION AND ASSAY MIXTURE ON ENZYME ACTIVITIES

Mn culture treatment (ppm)	Mn added in assay (M)	Tissue	
		Leaves	Roots
	Isocitric dehydrogenase		
0.00	0.000	2.3 *	20.0 *
0.25	0.000	2.5	33.0
10.00	0.000	33.0	334.0
	$LSD_{(5\%)}$	7.2	42.0
0.00	0.002	48.0	172.0
0.25	0.002	56.0	150.0
10.00	0.002	134.0	325.0
	$LSD_{(5\%)}$	26.0	38.0
	"Malic" enzyme		
0.00	0.000	1.6 *	23.0 *
0.25	0.000	2.7	26.0
10.00	0.000	31.0	129.0
	$LSD_{(5\%)}$	6.5	8.8
0.00	0.002	29.0	140.0
0.25	0.002	32.0	135.0
10.00	0.002	86.0	126.0
	$LSD_{(5\%)}$	22.0	11.0

* Units of activity expressed per mg protein.

TABLE II

EFFECT OF VARIABLE IRON AND MANGANESE CULTURE LEVELS ON ENZYME ACTIVITIES

Tissue	Fe culture treatment (ppm)	Mn culture treatment (ppm)		
		0.00	0.25	8.00
	Isocitric dehydrogenase			
Leaf	0.005	37 *	44 *	108 *
Leaf	0.250	40	51	108
Leaf	15.000	44	42	78
Leaf	25.000	41	39	66
Root	0.005	124	110	186
Root	0.250	128	122	192
Root	15.000	129	102	96
Root	25.000	102	102	96
	$LSD_{(5\%)}$ was 23 for leaves and 30 for roots			
	"Malic" enzyme			
Leaf	0.005	29 *	24 *	99 *
Leaf	0.250	23	33	104
Leaf	15.000	25	29	69
Leaf	25.000	22	27	59
Root	0.005	93	53	51
Root	0.250	100	103	70
Root	15.000	182	183	111
Root	25.000	192	170	102
	$LSD_{(5\%)}$ was 19 for leaves and 26 for roots			

* Units of activity expressed per mg protein.

Extracts of both roots and leaves of Mn-deficient plants contained as much isocitric dehydrogenase and "malic" enzyme as the extracts prepared from normal plants when assayed by the standard procedure which included Mn^{++}. The findings of Nason (11) in regard to the effect of Mn deficiency on isocitric dehydrogenase in tomato leaves are in agreement with these observations. Root and leaf extracts of Mn-toxic plants contained a 2- to 3-fold greater isocitric dehydrogenase activity than those of normal or Mn-deficient plants. Likewise, excessive Mn in culture solutions caused a 2.6-fold increase of "malic" enzyme of leaf extracts. Increased activity of this enzyme, however, was not observed with root extracts from these plants. Extracts of Mn-toxic plants did not contain an inhibitor of "malic" enzyme as indicated by experiments where equal amounts of extract of both Mn-toxic and normal roots were mixed and the results compared with the average of assays made on the two separate extracts. The specific activity of both enzymes was much greater in the extracts of roots than in those of leaves.

Since it is known that Mg^{++} at relatively high concentrations serves as an activator of isocitric dehydrogenase (1), one may argue that Mn-deficient plants contained enough Mg to activate the enzyme in vivo and therefore variable Mn in culture solutions did not influence the activity of the enzyme. To test this possibility plants were grown in media deficient in

both Mn and Mg and tissue extracts assayed for isocitric dehydrogenase. Again no significant differences were found when the results were compared with those of normal plants (data not presented).

FACTORS ANTAGONIZING INCREASED ENZYME ACTIVITIES: The results obtained from experiments with variable levels of Mn in culture solutions indicated that plants exhibiting symptoms of Mn toxicity were abnormally high in isocitric dehydrogenase and "malic" enzyme activities. In view of the reported antagonistic effect of Fe on Mn toxicity and vice versa (16) it was deemed necessary to study the effect of variable levels of both elements on these two enzymes. Bean plants were grown therefore with 3 levels of Mn and 4 levels of Fe in a complete factorial experiment as indicated in table II. The Fe and Mn deficiency treatments were initiated when seedlings were transplanted into solution cultures. Excessive concentrations of Fe and Mn were applied at the time of emergence of the second set of trifoliate leaves and the tissues were assayed 4 to 6 days later. Visual observations of the effects of these treatments on the plants indicated that high Fe treatments indeed prevented the severe symptoms of Mn toxicity that were observed at lower Fe levels. Manganese deficiency symptoms, however, were not visually influenced by levels of Fe in the media. This disagreement with the report of Somers and Shive (6) may be accounted for by the fact that Mn-deficiency symptoms were already apparent in those cultures that did not receive Mn before the two high levels of Fe were added. Also this experiment did not include Mn levels that resulted in incipient deficiencies as was the case with the experiments of Somers and Shive.

The isocitric dehydrogenase activity of leaves or roots and the "malic" enzyme activity of leaves from Mn-toxic plants with either deficient or normal Fe levels was greatly increased as compared with normal plants (table II). The increased enzyme activities associated with excessive Mn were nearly prevented if high levels of Fe (15 or 25 ppm) were added to cultures at the same time the high level of Mn was applied. The interaction of Fe and Mn as measured by "malic" enzyme activity of root extracts however showed an entirely different pattern. In this case Mn toxicity tended to reduce "malic" enzyme activity and high concentrations of Fe caused striking increases in it.

Other experiments were conducted to determine whether or not Fe was specific in its capacity to prevent increased enzyme activities of extracts of plants grown with excessive Mn. Studies were also made to test factors capable of decreasing the increased isocitric dehydrogenase and "malic" enzyme activities caused by previous Mn-toxic culture treatments. The influence of excessive Fe, Al, Cu, Mo, and Co on enzyme activities was also tested. In these experiments (table III) initial treatments were applied for 4 days following the emergence of the second set of trifoliate leaves. The culture solutions were then changed and various other treatments, referred to as "subsequent," were applied for the following 4 days, then enzyme assays were made.

TABLE III

EFFECT OF METAL TOXICITIES ON ENZYME ACTIVITIES

CULTURE TREATMENT *		ISOCITRIC DEHYDROGENASE		"MALIC" ENZYME	
INITIAL	SUBSEQUENT	LEAF	ROOT	LEAF	ROOT
ppm		*units per mg protein* **			
Normal	Normal	47	190	34	144
8 Mn	Normal	86	226	51	176
Normal	8 Mn	120	275	92	188
8 Mn	8 Mn	137	317	111	165
Normal	20 Fe	44	174	40	312
8 Mn	20 Fe	61	179	54	256
Normal	8 Mn + 20 Fe	66	162	63	230
8 Mn	8 Mn + 20 Fe	81	184	73	307
Normal	20 Al	56	197	30	219
8 Mn	20 Al	59	192	42	210
Normal	8 Mn + 20 Al	73	189	41	225
8 Mn	8 Mn + 20 Al	90	228	56	249
Normal	1 Cu	53	176	45	194
8 Mn	8 Mn + 1 Cu	142	294	...	...
Normal	20 Mo	58	231	37	157
Normal	5 Co	135	256	80	215
LSD(5%)		31	28	20	46

* Initial treatments were applied for 4 days after the appearance of the second set of trifoliate leaves. Subsequent treatments were then applied for 4 days and the plants assayed.

** Values are means of 2 experiments except the last 4 treatments which are means of 1.

Again extracts of leaves and roots of plants grown with toxic levels of Mn showed an increased isocitric dehydrogenase activity. "Malic" enzyme activity was also abnormally high in leaves of Mn-toxic plants but was not greatly elevated in roots of plants receiving this treatment. The increased enzyme activities due to excessive Mn were decreased to near-normal by transferring Mn-toxic plants to solutions containing high concentrations of either Fe or Al with a normal level of Mn. The increased enzyme activities also were prevented if high concentrations of Fe or Al were added at the same time that the toxic level of Mn was applied. High concentrations of both Fe and Al with normal levels of Mn caused large increases of "malic" enzyme activity of root extracts but had no effect on isocitric dehydrogenase activity of them. The enzyme activities were not greatly affected by toxic levels of Mo or Cu. Excessive Co markedly increased isocitric dehydrogenase and "malic" enzyme activities of root and leaf extracts.

IN VITRO METAL INHIBITION OF ENZYME ACTIVITIES: In all experiments conducted it was observed that both roots and leaves of plants grown with an excessive level of Mn in cultures were high in isocitric dehydrogenase and that leaves grown under these conditions were high in "malic" enzyme. The addition of high concentrations of Fe or Al to normal cultures did not significantly decrease enzyme activities. On the other hand, additions of Fe or Al to cultures receiving excessive Mn reduced or nearly prevented the

appearance of abnormal increases of isocitric dehydrogenase in roots and leaves and "malic" enzyme in leaves. In an effort to better understand the in vivo interaction of Mn versus Fe and Al an in vitro experiment was designed to study the possible inhibitory action of certain metals including Fe and Al on these enzymes. It has been reported that 5×10^{-5} M Cu^{++} inhibited by 50 % the isocitric dehydrogenase from pig heart (9).

A concentration of 2×10^{-4} M Al^{+++} or Fe^{+++} added to the standard assay inhibited isocitric dehydrogenase 36 and 48 %, respectively, whereas 6.7×10^{-4} M Cu^{++} only inhibited it 27 %. A Lineweaver-Burk plot of the inhibition of isocitric dehydrogenase by these metal ions with respect to Mn^{++} concentration is presented in figure 1 A. The ions tended to exhibit a noncompetitive type of inhibition with respect to Mn^{++}. The inhibition was not 100 % noncompetitive as evidenced by the failure of the lines to intersect at a common point when extrapolated to the abscissa (6). The Fe and Al ions were also found to exhibit a noncompetitive type of inhibition with respect to isocitrate and TPN^+ (data not presented). These metals apparently do not competitively compete with Mn^{++}, to any great extent, for its reaction site nor do they act by forming less active isocitrate or TPN^+ complexes. As shown in figure 1 B the "malic" enzyme also was noncompetitively inhibited by Fe^{+++} and Al^{+++}, but the inhibition was not as strong as that with

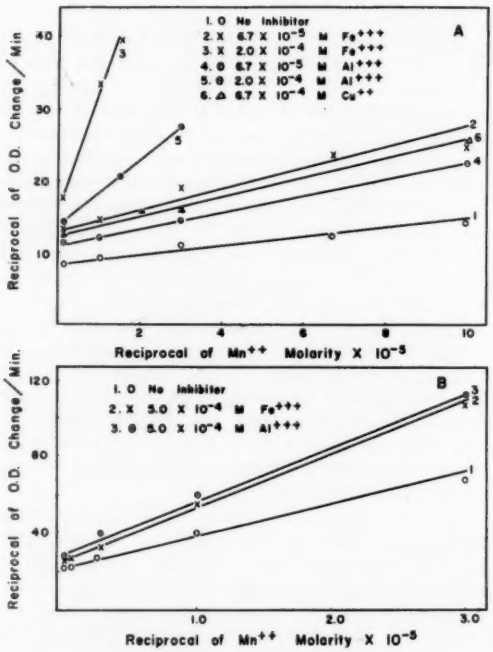
1. ○ No Inhibitor
2. X 6.7 X 10⁻⁵ M Fe+++
3. X 2.0 X 10⁻⁴ M Fe+++
4. ● 6.7 X 10⁻⁵ M Al+++
5. ● 2.0 X 10⁻⁴ M Al+++
6. △ 6.7 X 10⁻⁴ M Cu++
A
Reciprocal of O.D. Change / Min
Reciprocal of Mn++ Molarity X 10⁻⁵
1. ○ No Inhibitor
2. X 5.0 X 10⁻⁴ M Fe+++
3. ● 5.0 X 10⁻⁴ M Al+++
B
Reciprocal of O.D. Change / Min.
Reciprocal of Mn++ Molarity X 10⁻⁵

FIG. 1. Lineweaver-Burk plot of the inhibition of (A) isocitric dehydrogenase activity by Fe^{+++}, Al^{+++}, and Cu^{++} and (B) "malic" enzyme activity by Fe^{+++} and Al^{+++}.

isocitric dehydrogenase. The inhibitory effect of Fe^{++} could not be studied since it was rapidly oxidized to Fe^{+++} as characterized by absorption spectra studies.

EFFECT OF LACTIC DEHYDROGENASE ACTIVITY ON "MALIC" ENZYME ASSAYS: Effects of mineral element culture treatment on lactic dehydrogenase activity could influence the results of the "malic" enzyme assay since the products of the "malic" reaction (pyruvate and TPNH) lead to the regeneration of TPN^+ by lactic dehydrogenase. A test for lactic dehydrogenase activity of leaf and root extracts of plants grown in cultures that were Mn-deficient, normal, or toxic with either Mn, Fe, Cu, Al, Mo, or Co indicated that crude bean extracts contained no measurable TPNH–lactic dehydrogenase activity using the assay described above. Appreciable lactic dehydrogenase activity was observed, however, when a quantity of DPNH sufficient to saturate the enzyme was included.

DISCUSSION

Extracts from either normal or Mn-deficient plants showed little isocitric dehydrogenase or "malic" enzyme activity unless Mn^{++} was added in the assay. A possible explanation for the low activity without added Mn^{++} may be associated with the large dilution of extracts during preparation and assay. The soluble material of fresh roots was diluted 120-fold and that of leaves 240-fold in the assay media. Tissues of normal plants presumably contained sufficient Mn^{++} for enzyme activities in vivo but insufficient amounts to activate these enzymes after dilution in the in vitro assays.

The results indicating a Mn^{++} requirement for the isocitric dehydrogenase reaction differ from those reported by Ochoa (14) who found that extracts of an acetone powder of pig heart catalyzed a relatively rapid initial oxidation of isocitrate in the absence of Mn^{++} before an equilibrium was approached. This was interpreted as indicating that Mn^{++} was not required for the conversion of isocitrate to oxalosuccinate but was required in the decarboxylation of oxalosuccinate to alpha-ketoglutarate and CO_2. If the equilibrium of the dehydrogenation reaction is toward oxalosuccinate as reported by Ochoa (14) and as recalculated by Burton and Krebs (3), one would expect that a considerable amount of dehydrogenation would take place in the absence of Mn^{++} before an equilibrium was reached with oxalosuccinate. Since isocitric dehydrogenase of bean extracts was practically inactive without added Mn^{++} it is possible that Mn^{++} was required for the dehydrogenation step, as well as the decarboxylation step. Perhaps a more reasonable explanation is that the extracts catalyzed a one-step oxidative decarboxylation of isocitrate and that no free oxalosuccinate was formed. If the latter mechanism is correct then the oxidative decarboxylation of isocitrate is analogous to the "malic" enzyme reaction (15).

Since extracts of Mn-deficient plants contained as much isocitric dehydrogenase and "malic" enzyme as those of normal plants it appears that Mn deficiency

had no specific effect on the synthesis of the protein moieties of these enzymes. Under the condition of Mn toxicity on the other hand large increases of enzyme activities were observed with or without the addition of Mn^{++} to the assay mixture. Apparently an increased synthesis of both isocitric dehydrogenase and "malic" enzyme was induced by excessive Mn in culture solution.

An increased isocitric dehydrogenase activity of root and leaf tissues and an increased "malic" enzyme activity of leaves was associated with excessive Mn in culture solution. The addition of high concentrations of Fe or Al either with the toxic level of Mn or after the addition of excessive Mn resulted in enzyme activities in the same range of magnitude as those of normal tissues. It may be assumed that the high enzyme activity associated with Mn toxicity was evidence of some metabolic unbalance resulting from excesses of this element. Apparently large amounts of Fe or Al applied to Mn-toxic cultures either reduced the synthesis of "malic" and isocitric dehydrogenase or inactivated these enzymes after they were formed. The inhibition experiments indicated that Fe^{+++} and Al^{+++} do not compete with Mn^{++} for the same active sites on the enzyme surfaces but instead inactivate the enzymes by combining with them at some other site.

The antagonism of the increased isocitric dehydrogenase activity of Mn-toxic leaf and root tissues by large amount of Fe in culture solutions complements the well known Fe-Mn relationship on growth (16). The similar antagonistic effect of these metals on the "malic" enzyme activity of leaves would likewise help explain the Fe-Mn interaction. The effect of excessive Mn or Fe on "malic" enzyme in roots was almost completely opposite to the trend observed with this enzyme in leaves. Although these effects of Fe and Mn also were antagonistic, they make the interpretation of the mechanism of action of these metals on enzyme constitution difficult.

Summary

Tissue extracts from Mn-deficient or normal plants catalyzed very little dehydrogenation of isocitrate or malate unless Mn^{++} was added to the assay reaction mixture. Extracts of Mn-toxic plants, however, showed considerable dehydrogenase activities without exogenous Mn^{++}.

When assayed with Mn^{++} in the reaction mixture, Mn-deficient tissues were found to contain as much isocitric dehydrogenase and "malic" enzyme as the tissues of normal plants. Leaf and root extracts of Mn-toxic plants showed a 2- to 3-fold increased isocitric dehydrogenase activity. This increased activity was decreased to near-normal by adding high concentrations of Fe or Al to culture solutions of Mn-toxic plants or was nearly prevented if large amounts of Fe or Al were added at the same time as the toxic concentration of Mn was applied.

Metal ions affected the "malic" enzyme activity of leaves in the same way as they affected isocitric dehydrogenase activities of leaves and roots; however, an almost reverse relationship of the metals on "malic" enzyme activity of root extracts was found. The effect of some other metal toxicities and deficiencies on the activities of these enzymes is reported.

With respect to Mn^{++}, isocitric dehydrogenase and "malic" enzyme activities were noncompetitively inhibited by low concentrations of Fe^{+++} and Al^{+++} and by higher amounts of Cu^{++}. The inhibition was mainly of a noncompetitive nature but showed some tendency to influence the dissociation of the enzyme-Mn^{++} complex.

The relationship of metal ion culture level to isocitric dehydrogenase and "malic" enzyme constitution is discussed in connection with the Fe-Mn interaction on growth.

Literature Cited

1. Adler, E., Euler, H., Gunther, G., and Plass, Marianne. Isocitric dehydrogenase and glutamic acid synthesis in animal tissue. Biochem. Jour. 33: 1028–1045. 1939.
2. Brown, J. C. and Steinberg, R. Iron and copper enzymes in leaf lamina of tobacco when deficient in micronutrients or grown on calcareous and organic soils. Plant Physiol. 28: 488–494. 1953.
3. Burton, K. and Krebs, H. A. The free-energy changes associated with the individual steps of the tricarboxylic acid cycle, glycolysis, and alcoholic fermentation and with hydrolysis of the pyrophosphate groups of adenosine triphosphate. Biochem. Jour. 54: 94–107. 1953.
4. Ceithaml, J. and Vennesland, Birgit. The synthesis of tricarboxylic acids by carbon dioxide fixation in parsley root preparations. Jour. Biol. Chem. 178: 133–143. 1949.
5. Conn, E., Vennesland, Birgit, and Kraemer, L. M. Distribution of a triphosphopyridine nucleotide–specific enzyme catalyzing the reversible oxidative decarboxylation of malic acid in higher plants. Arch. Biochem. 23: 179–197. 1949.
6. Friedenwald, J. S. and Maengwyn-Davies, Gertrude. Elementary kinetic theory of enzymatic activity, first order theory. In: The Mechanism of Enzyme Action, W. D. McElroy and B. Glass, Ed. Pp. 154–179. The Johns Hopkins Press, Baltimore, Maryland. 1954.
7. Kraemer, L. M., Conn, E. E., and Vennesland, Birgit. The β-carboxylases of plants. III. Oxalacetic carboxylase of wheat germ. Jour. Biol. Chem. 188: 583–591. 1951.
8. Healy, W. B., Cheng, Sze-chuh, and McElroy, W. D. Metal toxicity and iron deficiency effects on enzymes in Neurospora. Arch. Biochem. Biophys. 54: 206–214. 1955.
9. Lotspeich, W. D. and Peters, R. A. The action of sulfhydryl inhibitors upon isocitric dehydrogenase with especial reference to the behavior of some trivalent arsenicals. Biochem. Jour. 49: 704–709. 1951.
10. Lowry, O. H., Rosebrough, Nira J., Farr, A. L., and Randall, Rose J. Protein measurement with the Folin phenol reagent. Jour. Biol. Chem. 193: 265–275. 1951.
11. Nason, A. Metabolism of micronutrient elements in higher plants. II. Effect of copper deficiency on the isocitric enzyme in tomato leaves. Jour. Biol. Chem. 198: 643–653. 1952.

12. NASON, A., KAPLAN, N. O., and OLDEWURTEL, H. A. Further studies of nutritional conditions affecting enzymatic constitution of Neurospora. Jour. Biol. Chem. 201: 435–444. 1953.
13. NICHOLAS, D. J. D., NASON, A., and MCELROY, W. D. Molybdenum and nitrate reductase. I. Effect of molybdenum deficiency on the Neurospora enzyme. Jour. Biol. Chem. 207: 341–351. 1954.
14. OCHOA, S. Biosynthesis of tricarboxylic acids by carbon dioxide fixation. III. Enzymatic mechanisms. Jour. Biol. Chem. 174: 133–157. 1948.
15. SALLES, J. B. V. and OCHOA, S. Biosynthesis of dicarboxylic acids by carbon dioxide fixation. II. Further study of the properties of the "malic" enzyme of pigeon liver. Jour. Biol. Chem. 187: 849–861. 1950.
16. SOMERS, I. I. and SHIVE, J. W. The iron-manganese relation in plant metabolism. Plant Physiol. 17: 582–602. 1942.
17. STOUT, P. R. and ARNON, D. I. Experimental methods for the study of the role of copper, manganese, and zinc in the nutrition of higher plants. Amer. Jour. Bot. 26: 144–149. 1939.
18. WEINSTEIN, L. H. and ROBBINS, W. R. The effect of different iron and manganese nutrient levels on the catalase and cytochrome oxidase activities of green and albino sunflower leaf tissues. Plant Physiol. 30: 27–32. 1955.

THE ABSORPTION AND TRANSLOCATION OF SULFUR IN RED KIDNEY BEAN[1,2,3]

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The principal objective of this study is to determine the characteristics of translocation and the ultimate partition of sulfur between the various parts of Red Kidney bean plants. The methods employed are those of geographic tracing by means of radioactive sulfur. Data are presented on the following points: 1) the effect of nutrient sulfur content on dry matter yield and on sulfur concentration within organs; 2) the effect of pH on sulfur absorption; 3) the exchange of sulfur between various organs; 4) the rate of downward translocation of sulfur in stems.

The justification for such a study lies in the fact that only a few papers bearing on the translocation of sulfur have appeared. Rippel (13) states that in various deciduous trees there is no migration of sulfur from regions of storage to new tissues as growth begins in the spring. He classes sulfur with calcium, as a "stabile elemente." Marsh (9) also states that sulfur resembles calcium in that it is not mobilized to any great extent. Little was withdrawn from leaves of the apple prior to leaf fall. Wood and Barrien (22) found that during sulfur starvation protein sulfur decreased and SO_4 sulfur increased in grass leaves held in darkness. Sulfate was not translocated to stems and roots as were the amino acids. Wood (19) concludes that in fully matured plants sulfate is relatively immobile in leaves.

In the work of Thomas et al (16), using radioactive sulfur, there is presented definite evidence that sulfur is conducted from one region to another as it is needed for growth, being conducted as the sulfate ion. They have also shown (17) that sulfate absorbed through leaves of alfalfa plants can be translocated to the crowns and then into untreated stems.

The data presented herein add specifically to the information on the translocation and partition of sulfur between the various plant parts during the early phases of growth.

METHODS

Red Kidney bean (commercial stock) served as experimental material. The plants were grown in a Hoagland type nutrient solution as follows: $Ca(NO_3)_2$, 0.0025 M; KNO_3, 0.0025 M; KH_2PO_4, 0.0005 M; $MgSO_4$ or $MgCl_2$ to give both 0.0010 M Mg and varying amounts of sulfate sulfur, and micronutrients according to Arnon (1). Growth conditions were as follows: temp 23° ± 1° C; R.H. 60 % ± 2.5 %; light, artificial at 1000 to 1200 fc (2 daylight plus 10 soft white fluorescent tubes); aeration through sintered glass; and hydrogen ion concentrations maintained at approximately pH 6. No significant variation in absorption, translocation and partition of sulfur was observed over the pH range of 4 to 7. The least pH drift occurred at 6.

The total sulfur was determined for roots, stems including the hypocotyl and petioles, primary leaves, and trifoliate leaves (both less petioles). The composited similar parts of six plants, all grown in the same tank, constituted the samples. Each experiment, except the one represented in figure 1 D was replicated at least once. Duplicate determinations were made when sample size permitted.

In order to render the sulfur of seed origin distinguishable from sulfur of nutrient solution origin after absorption by the plant, S^{35} was added to the nutrient sulfur. This will be referred to as labeeld sulfur, or indicated by S*. Prior to use the number of disintegrations per minute per 15 mgs of BaS^*O_4 from the nutrient solution was determined. This value is designated by "a." At the termination of the growth period, during which time the labeled sulfur was being absorbed by the plants and diluted with seed sulfur already present, 15 mgs of the BaS^*O_4

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[2] The radioisotopes were acquired from U. S. Atomic Energy Commission, Oak Ridge, Tennessee.
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derived from each plant sample was analyzed for S^{35}. This value in disintegrations per minute per 15 mgs BaS^*O_4 was corrected for decay during the experiment and the value designated by "b." Then since "a" represents the original S^{35} per unit of total sulfur and "b" the final S^{35} per unit of total sulfur, the percentage composition of nutrient sulfur in each plant part is $b/a \times 100$. The total labeled sulfur and the total sulfur in each part was determined from the yield of $BaSO_4$ from each part. Oxidation of the samples was accomplished in an oxygen bomb.

Results

The Effect of Nutrient Sulfur Content and of pH on Dry Matter Yield and Sulfur Concentration: The plants grown with no nutrient sulfur, i.e., only seed sulfur, showed severe signs of sulfur deficiency, the most pronounced of which were a marked reduction in growth of trifoliate leaves with yellowing and necrotic spotting. The roots remained quite normal in appearance. A comparison of the dry matter yield of plants grown at several nutrient sulfur levels is made in figure 1 A. Sulfur, it can be seen, was not limiting at nutrient concentrations of 0.125 millimolar, or above, under the conditions of these experiments, when one liter of nutrient solution per plant was renewed each four days. At concentrations above this value absorption was essentially independent of concentration. The uniformity in the dry matter yield of each part between the nutrient sulfur concentrations of 0.125 and 1.0 millimolar indicates that 0.03 millimoles of sulfur per plant per day is adequate during the initial 12 days of growth.

There was observed a lack of uniformity in the growth response of the primary leaves which was considered to have more to do with the degree of injury suffered during the shedding of the seed coat than with the nutrient treatments. The extent of injury could not always be ascertained at the time the seedlings were selected for further use.

The amount of sulfur contained in each plant part at harvest time is shown in figure 1 B. There was a slight tendency for the sulfur content of the roots to increase with increasing amounts of nutrient sulfur so that on a concentration basis, i.e., mg S/gm dry matter (fig 1 C), it seems justifiable to conclude that a slightly ascending relationship existed. This could be due to retention of nutrient solution on the root surfaces. Roots and trifoliate leaves were higher in sulfur concentrations than stems and primary leaves. However, the roots did not adsorb sulfur as phosphorus and iron are sometimes adsorbed (Rediske and and Biddulph, 12).

There was very little variation in dry matter yield or in sulfur content of the plants as a whole, or of their constituent parts, when grown at pH values ranging from 4 to 7. Two replications of six plants per unit served as the basis for this statement. These plants were grown in the greenhouse, which was not subjected to as close a temperature, light, or humidity control as was the case for the plants represented in figure 1.

The percentage of the total tissue sulfur which was derived from each of the two sources, i.e., the seed and the nutrient solution, is shown for each plant part in figure 1 D.

The incoming sulfur of nutrient origin, which was marked with S^{35}, becomes diluted with sulfur of seed origin which had been deposited before the nutrient sulfur was made available. The time chosen for beginning the administration of the nutrient sulfur corresponded to the stage of development at which the hypocotyl had straightened, the primary leaves were well expanded and the root system fairly well formed. From this time onward growth was more rapid in the root, stem, and especially the trifoliate leaves. At the time of harvest the sulfur of nutrient origin in the various parts was approximately as follows: primary leaves 50 %, stems 66 %, roots 75 % and trifoliate leaves 82 %. Sulfur of seed origin, of course, made up the remainder (fig 1 D).

At the lowest nutrient sulfur levels the roots retained relatively more seed sulfur than at the higher levels. It is also true that the roots of those plants grown on seed sulfur only were heavier than those grown with additions of nutrient sulfur (fig 1 A). To make doubly sure of this point this part of the experiment was repeated with similar results being obtained. The trifoliate leaves of these plants were very limited in size and number and manifest severe sulfur deficiency symptoms. This is all indicative of the ability of the root to acquire seed sulfur early and retain it from the developing trifoliate leaves even to their death from sulfur deficiency. A corresponding amount of organic seed reserves was also acquired and retained to facilitate the root extension characteristic of this metabolic condition. It is necessary to assume some mechanism operating within the tissue systems which ensures the development of the root system, even at the expense of the aerial parts, when sulfur is a limiting factor.

From these results it is apparent that sulfur of seed and nutrient solution origin will not become uniformly distributed throughout the plant. The rate of turnover of sulfur compounds within tissues is too low, as is shown by the fact that after 12 days with the marked sulfur available to the root the primary leaves have fallen to only 50 % seed sulfur while the newly developed trifoliate leaves have risen to only 18 % seed sulfur. A time sequence study would be required to fully establish a turnover rate.

The extent of movement of labeled sulfur, acquired at an earlier period, into current growth made during a time when no labeled sulfur was available, can be seen by comparing the autoradiographs A and B of figure 2. Figure 2 A shows the distribution of S^{35} in the plant after the roots had been immersed in a nutrient solution containing the radioisotope for four days (from the fourth to the eighth day after the hypocotyl became erect). Figure 2 B shows a plant treated similarly but allowed four additional days of

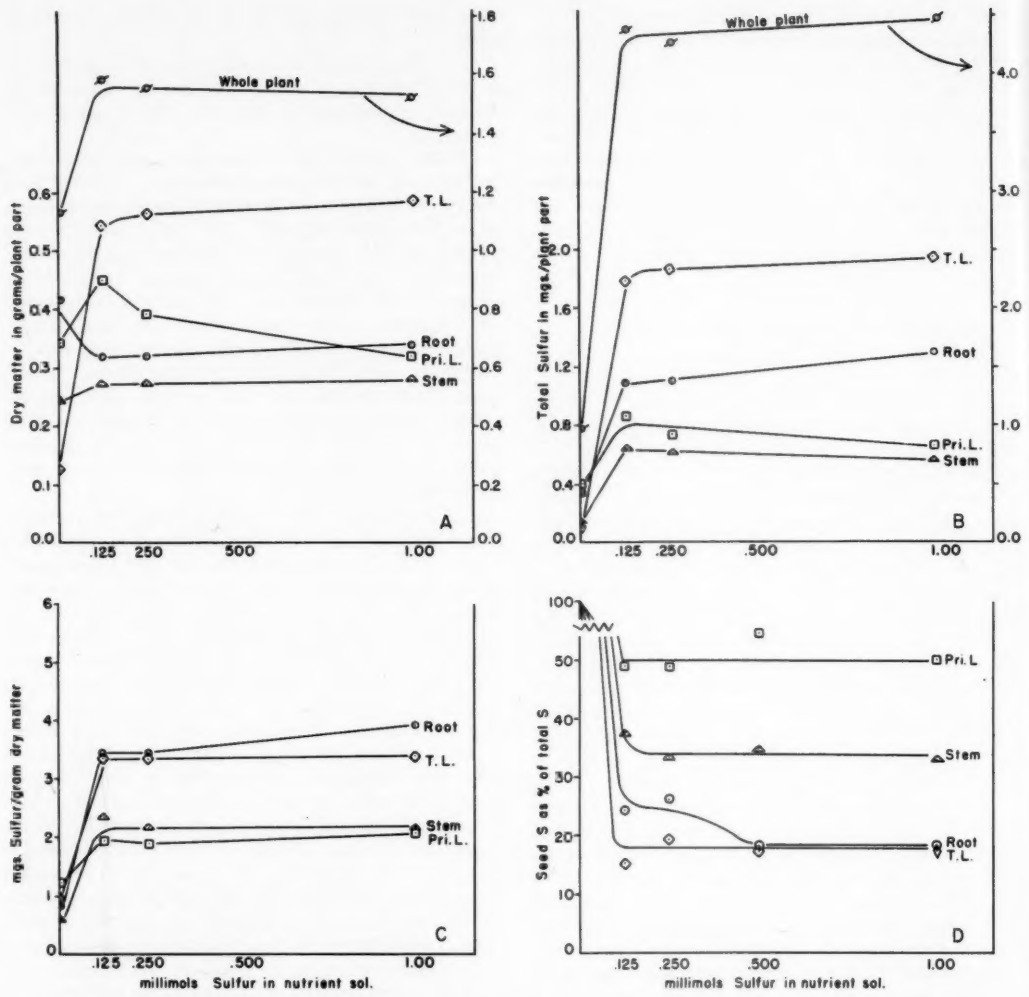

FIG. 1. A. The dry matter yield of separate plant parts, shown on the left ordinate, and of the whole plant, shown on the right ordinate, as affected by the varying nutrient sulfur concentrations shown on the abscissa.

B. The total sulfur content of separate plant parts, shown on the left ordinate, and of the whole plant, shown on the right ordinate, as affected by the varying nutrient sulfur concentrations shown on the abscissa.

C. The sulfur concentration (mg S/gm dry matter) of separate plant parts as affected by the varying nutrient sulfur concentrations shown on the abscissa.

D. The percentage of sulfur of seed origin, and conversely the percentage of sulfur of nutrient solution origin, in separate plant parts as affected by the nutrient sulfur concentrations shown on the abscissa.

growth in a nutrient solution containing no radioactive sulfur. A comparison of A and B (fig 2) shows that labeled sulfur from the root and older leaves moves into the young leaves which expand during the period when no labeled sulfur is available to the root. Since the bean root does not adsorb large amounts of sulfur it is evident that the S^{35} found in the youngest leaves must have been translocated from other parts within the plant.

The nutrient solutions employed for the whole period were of the Hoagland type mentioned earlier; so no abnormal nutritional conditions existed during any part of the experimental period. The plants were also grown under the controlled conditions listed earlier. One gets the impression from these autoradiographs that a part of the nutrient sulfur, after its absorption, remains mobile and circulates rather freely within the plant. Of the mobile fraction being moved upward by the xylem a portion will be trapped in organic synthesis, particularly in young

leaves and stem tips, while a significant portion of that normally received by mature leaves may be free to circulate. In this manner the younger leaves and the buds may be continuously supplied with sulfur, whereas under conditions of scant supply to the roots the older tissues may become deficient. This is in keeping with our observations that the older trifoliate leaves are the first to show sulfur deficiency under limited sulfur nutrition. Sulfur, in the bean, behaves like phosphorus (2) in the sequence of development of visual deficiency symptoms on leaves of different ages and positions. This is similar to the sequence in tomato (11), but contrary to that for soybean (4), black mustard (6), tea (15) and tobacco (10). Sunflower showed no gradation (5).

THE DOWNWARD TRANSLOCATION OF SULFUR IN THE STEM: A more direct method for determining the mobility of sulfur within the bean plant was used to supplement the work above. This method made possible the determination of the rate of downward movement of sulfur in the stem, and furnished as well some information on the distribution pattern of the mobile sulfur in the stem.

Twelve-day-old plants, grown as above, were used. The S^{35} was introduced into the leaf either by the leaf flap method as described earlier (Biddulph, 2) or by spraying the material directly onto the surface of the leaf. The S^{35} was carrier free and was adjusted to a pH of 4 prior to use. It assayed approximately 1 mc/ml and 50 microliters of solution were used per treatment. The treatments were begun at 2 P.M. and the migration period terminated 30 or 60 minutes later when the roots and the tops above the point of insertion of the first trifoliate leaf (the treated leaf) were simultaneously cut off. Each of the included stems was then divided into one-inch sections for individual S^{35} assays using a wet digestion procedure. In two of the trials the solution to be injected contained sucrose at a concentration of 2.5 %. This was to determine the possible effect of an enhanced sugar supply in the leaf on accelerating the translocation of sulfur. There was no appreciable influence manifest when sucrose was applied by this method. Higher sugar concentrations were tried but they invariably lowered the amount of the radioisotope which entered the leaf, and caused wilting in the vicinity of the leaf flap. When the petiole of the leaf was steamed just prior to the S^{35} application no movement of S^{35} occurred from the leaf. The results of the experimental work for this section are shown in table I.

During the thirty minute migration period the S^{35} had moved from the leaf into the root, the crown of which was 8 inches below the pulvinus of the treated leaflet. In two of the four cases reported on in table I this amounted to a rate of movement well in excess of 40 cm/hr. The lowermost section showed a relatively high activity, indicating that the front of the marked S^{35} had entered the root sometime earlier.

Figure 2 C is an autoradiograph of a bean plant treated as above (S^{35} supplied to a leaflet) except that a 24-hour migration period was allowed. It is evident that the S^{35} has moved throughout the whole plant. As a result of a rather extensive experiment it was found that such nutritional factors as high or low sulfur levels, i.e., 0.0010 and 0.00001 M, high and low calcium levels, i.e., 0.0050 and 0.0005 M, and the pH values of 4 and 7, in all possible combinations did not significantly alter the rate, amount or pattern

FIG. 2. A. S^{35} autoradiograph of a bean plant receiving 8.2 μc S^{35}/liter of nutrient solution (at pH 6) during the four days prior to harvest. Exposure period 90 days, to Eastman no-screen x-ray film.

B. S^{35} autoradiograph of a bean plant treated as in A but allowed four additional days of growth while no tracer was present in the nutrient medium. Exposure 90 days, to Eastman no-screen x-ray film.

C. S^{35} autoradiograph of a bean plant, the lowermost trifoliate leaf of which had been treated with 28.1 μc of S^{35}/50 μl of solution at a pH of 4. The treated leaflet is indicated by an arrow. A migration period of 24 hours was allowed and the exposure time was 60 days, to Eastman no-screen x-ray film.

TABLE I

THREE EXPERIMENTS TO SHOW THE DISTRIBUTION OF S^{35} IN THE BEAN STEM BELOW THE NODE OF THE TREATED LEAF

STEM SECTION	EXPT 1 LEAF INJECTION			EXPT 2 LEAF INJECTION		EXPT 3 SPRAY
	S^{35}	S^{35} + SUCROSE	PETIOLE STEAMED, S^{35}	S^{35}	S^{35} + SUCROSE	S^{35}
1	30,650	34,750	0	9,150	6,430	210,000
2	12,640	16,900	0	2,378	1,240	146,400
3	6,780	9,800	0	862	513	100,000
4	4,520	5,910	0	484	389	55,800
5	1,917	2,000	0	92	trace	44,500

The concentration of the S^{35} used for treatment varied between experiments but not within them. All solutions were at pH 6. Sucrose, when used, was 2.5 %. Solutions were applied to the lowest trifoliate leaf. Sprayed areas were 1 inch in diameter and on the lower surface. Migration periods: Experiments 1 and 2—30 min, experiment 3—60 min. Data are in counts per min (cpm) per one-inch section, except for the #5 sections which varied in length from 1 to 1.5 inches. Section 1 is nearest the treated leaf. The data are for single plants.

of S^{35} movement. By contrast the movement of iron can be profoundly influenced by phosphorus nutrition levels, when introduced by the same method (Rediske and Biddulph, 12).

DISCUSSION

Under the growth conditions imposed upon the plants herein studied, the dry matter yield failed to increase as the plants were subjected to nutrient sulfur concentrations beyond 0.125 millimolar. Thus the Red Kidney bean behaves very differently from the cotton plants reported on by Ergle and Eaton (8). They found increasing yields as the nutrient sulfur concentration was raised from zero to two millimolar, but the rate of increase was considerably reduced bepond the nutrient concentration of 0.50 millimolar as though a plateau were being approached.

In the kidney bean sulfur absorption was independent of nutrient sulfur concentration between the ranges of 0.125 and 1.0 millimolar. This again is in contrast to the cotton plants described above wherein sulfur absorption was shown to be quite strictly a function of nutrient sulfur concentration to the limit studied, namely, 2.0 millimolar.

One additional point of contrast between the kidney bean and cotton was found when comparing the sulfur concentration of tissues. In cotton the percentage of sulfur in leaves and stalks increases with increasing nutrient sulfur concentration to an extent which led Ergle and Eaton (8) to remark that "the tendency for the cotton plant to accumulate sulfate in its leaf cells (but not in stem cells) seems quite remarkable." In the bean the concentration of sulfur did not increase in any tissue, with the possible exception of the roots, in nutrient sulfur concentrations above 0.125 millimolar. This strict proportionality between sulfur and dry matter yield seems worthy of special mention.

A very interesting relationship among the various parts of the plant is shown in the competition among them for the sulfur contained in the seed. The first export is downward from the cotyledons through the hypocotyl to the developing root system, then upward to the primary leaves. The further development of the epicotyl is somewhat delayed, and the root will release a portion of its sulfur of seed origin to it only when sulfur from the root environment becomes available. The aerial parts, particularly the trifoliate leaves, gain an amount corresponding to the loss by the root. The consumption of the seed sulfur by root and primary leaves results in the upper part of the epicotyl being particularly low in this material as extensive tissue elaboration begins; so sulfur deficiency symptoms develop early in these upper parts. The seed sulfur within the root cells is held tenaciously and little is re-exported until such time as sulfur becomes available by absorption from the root environment. This incoming sulfur presumably allows a limited amount of turnover of sulfur compounds of seed origin which are retained by the root under conditions of sulfur deficiency. Eaton's data (4, 5, 6) led him to conclude that there was little evidence of protein breakdown and reutilization of sulfur under conditions of sulfur starvation in soybean, sunflower and black mustard. Apparently the protein bound sulfur is comparatively stable and not subject to extensive turnover. The conditions under which breakdown of sulfur containing proteins occur have been investigated by Wood and Barrien (20, 21, 22).

The normal activities of a rapidly growing bean plant under conditions of at least a moderate level of sulfur nutrition include an export of sulfur from the lower mature leaves to the root. As the transpiration stream delivers the sulfur to the leaves, there occurs a continuous though somewhat limited circulation of sulfur within the plant. In this way the concentration of sulfur in the leaves is maintained relatively constant and excesses, such as were reported by Ergle and Eaton (8) for cotton do not occur. Export from immature leaves is extremely low or nonexistent, as that which is acquired from the root, or by external application, is used locally in tissue elaborations.

As to the amount of sulfur normally translocated in beans it is greater than the translocation of calcium, which is nil, and iron, which is significant but dependent on certain nutritional factors (12) and compares favorably with phosphorus translocation, which is appreciable (2). Sulfur is freely mobile under a wide variety of nutrient conditions. When aplied to a bean leaf, either by injection or by spray application, it can be detected in all parts of the plant within thirty minutes. The amount translocated is adequate to supply at least a significant portion of the needs of the various tissues. In short, it would appear that sulfur could be at least as effectively utilized from foliar applications as is phosphorus (7, 14).

This means that foliar application could adequately supplement soil absorbed sulfur, especially where soil sulfur is limiting.

The rate of downward translocation of sulfur, shown to take place *in the phloem* (3), compares favorably with our values for P^{32}, C^{14} labeled sucrose, Fe^{55} and THO (tritiated water), each of which appears to be subject to some variation. However, this variation in rate is much less than the variation in the amounts of the different elements being translocated. The concentration of the mobile fraction in the stem is logarithmic with respect to distance traveled and is the same as would be expected if movement were by a diffusion process. The same distribution pattern has been found for mobile sulfur, phosrphorus, sucrose, iron, zinc and water, but the probable significance of these data cannot be discussed from the sulfur data alone. The resemblance of the distribution pattern to that which would be expected of a diffusion process is not, in itself, sufficient evidence to rule out any of the other theories of movement.

Summary

The data show 1) that there is a rather constant relationship between dry matter yield and sulfur content of each plant part throughout the nutrient range of 0.125 and 1.0 millimolar sulfur, 2) that trifoliate leaves and roots are richer in sulfur than stems and primary leaves, 3) that pH has little effect on sulfur uptake, 4) that a portion of the total sulfur within the plant remains mobile and moves freely from one organ to another, 5) that a portion of the seed sulfur originally translocated to the roots is released for upward translocation only when sulfur enters the plant from the root environment, and 6) that the rate of downward movement of sulfur in the phloem of the stem is similar to that for phosphorus and sucrose and exceeds 40 cm/hr.

Literature Cited

1. Arnon, D. I. Microelements in culture-solution experiments with higher plants. Amer. Jour. Bot. 25: 322–325. 1938.
2. Biddulph, O. Diurnal migration of injected radiophosphorus from bean leaves. Amer. Jour. Bot. 28: 348–352. 1941.
3. Biddulph, Susann F. Visual indications of S^{35} and P^{32} translocation in the phloem. Amer. Jour. Bot. (In press.)
4. Eaton, S. V. Influence of sulfur deficiency on metabolism of the soybean. Bot. Gaz. 97: 68–100. 1935.
5. Eaton, S. V. Influence of sulfur deficiency on metabolism of the sunflower. Bot. Gaz. 102: 536–556. 1941.
6. Eaton, S. V. Influence of sulfur deficiency on metabolism of black mustard. Bot. Gaz. 104: 306–315. 1942.
7. Eggert, R., Kardos, L. T., and Smith, R. D. The relative absorption of phosphorus by apple trees and fruits from foliar sprays, and from soil applications of fertilizer, using radioactive phosphorus as a tracer. Proc. Amer. Soc. Hort. Sci. 60: 75–86. 1952.
8. Ergle, D. R. and Eaton, F. M. Sulfur nutrition of cotton. Plant Physiol. 26: 639–654. 1951.
9. Marsh, R. S. Seasonal variation of sulfur content in certain tissues of the apple tree. Bot. Gaz. 75: 400–413. 1923.
10. McMurtrey, J. E. Symptoms of field-grown tobacco characteristic of the deficient supply of each of several essential chemical elements. U. S. Dept. Agr. Tech. Bull. 612. 1938.
11. Nightengale, G. T., Schermerhorn, L. G., and Robbins, W. R. Effect of sulfur deficiency on metabolism in tomato. Plant Physiol. 7: 565–595. 1932.
12. Rediske, J. H. and Biddulph, O. The absorption and translocation of iron. Plant Physiol. 28: 576–593. 1953.
13. Rippel, A. Untersuchungen über die Mobilisation der Aschenbestandteile und des Stickstoff in Zweigen biem frühjahruchen Austreiben. Biochem. Zeits. 113: 125–144. 1921.
14. Silberstein, O. and Wittwer, S. H. Foliar application of phosphatic nutrients to certain vegetable crops. Proc. Amer. Soc. Hort. Sci. 58: 179–190. 1951.
15. Storey, H. H. and Leach, R. A sulfur deficiency disease of the tea bush. Annals Appl. Biol. 20: 23–56. 1933.
16. Thomas, M. D., Hendricks, R. H., Bryner, L. C., and Hill, G. R. A study of the sulphur metabolism of wheat, barley and corn using radioactive sulphur. Plant Physiol. 19: 227–244. 1944.
17. Thomas, M. D., Hendricks, R. H., and Hill, G. R. Sulfur metabolism in alfalfa. Soil Sci. 70: 19–26. 1950.
18. Vernon, L. P. and Arnoff, S. Metabolism of soybean leaves. IV. Translocation from soybean leaves. Arch. Biochem. Biophys. 36: 383, 398. 1952.
19. Wood, J. G. Metabolism of sulfur in plants. Chronica Botanica 7: 1–4. 1942.
20. Wood, J. G. and Barrien, B. S. Studies on the sulphur metabolism of plants. I. Preliminary investigations on the effects of different external concentrations of sulphate, ammonia and cystine on the amount of sulphur-containing compounds in leaves. New Phytologist 38: 125–149. 1939.
21. Wood, J. G. and Barrien, B. S. Studies on the sulphur metabolism of plants. II. The effects of nitrogen supply on the amounts of protein sulphur, sulphate sulphur and on the value of the ratio of protein nitrogen and protein sulphur in leaves at different stages during the life cycle of the plant. New Phytologist 38: 257–264. 1939.
22. Wood, J. G. and Barrien, B. S. Studies on the sulphur metabolism of plants. III. On changes in amounts of protein sulphur and sulphate sulphur during starvation. New Phytologist 38: 265–272. 1939.

STUDIES IN PLANT METABOLISM. VI. EFFECT OF 2,4-D ON THE METABOLISM OF ASPARTIC ACID AND GLUTAMIC ACID IN THE BEAN PLANT[1,2,3]

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In studying the effect of 2,4-D treatment on the chemical composition of bean plants, Sell et al (7) reported that the stem of 2,4-D treated plants contained almost twice as much crude protein as do stems of control plants. Later, Weller et al (9) found that 2,4-D treatment caused a slight decrease in crude leaf protein while the crude root protein remained approximately the same. The amino acid composition in the crude protein from 2,4-D treated stems were different from those of the control plants. A small difference was noted in the leaf and root proteins. These data suggested to the authors that a change had occurred in the character of the protein. Using potato tubers, Payne et al (5) found that 2,4-D caused an increase in free glutamic acid and a decrease in eleven amino acids including aspartic acid. The decrease was explained as an increased catabolism of the free amino acids. In a preliminary investigation on the effects of 2,4-D 2,4,5-T and indoleacetic acid treatment, we have found that the amounts of total aspartic and glutamic acids decreased in both the stems and roots of 2,4-D and 2,4,5-T treated plants, with the greatest change occurring in the stems. Two possible explanations may be given: 1) the 2,4-D treatment might inhibit the synthesis of these amino acids or 2) this treatment might increase the rate of oxidation of the amino acids.

In order to clarify this point, the present investigation, using carbon 14 as a tracer to study the rates of synthesis of both glutamic acid and aspartic acid in 2,4-D treated and control plants was carried out.

MATERIALS AND METHODS

TREATMENT OF PLANTS: Bean plants (*Phaseolus vulgaris* var. Black Valentine) were grown in potted soil, four plants to a pot, under greenhouse conditions. The plants were allowed to grow until the primary leaves were fully unfolded and the second internode well defined. At this time three uniform pots were chosen and two plants in each pot were treated on the midrib of one primary leaf with 50 μgm of 2,4-D dissolved in an ethanol solution containing 0.1 % of the free 2,4-D acid and 0.5 % Tween-20. The other two plants in each pot were left untreated as control plants.

A closed system was prepared as shown in figure 1. This system consisted of a bell jar large enough to contain one set of four plants, a column of calcium chloride to absorb the excess moisture, a generator to produce radioactive CO_2 from $BaC^{14}O_3$ after the system was sealed, a Geiger-Müller tube to detect the radioactivity, and a pump to circulate the atmosphere.

One day after treatment, the first set of bean plants was placed in the closed system and exposed to an atmosphere containing $C^{14}O_2$ for a period of 12 hours in light and 12 hours in darkness. Two 40-watt fluorescent lamps which have a light intensity of 200 fc were set 6 inches from the bell jar on opposite sides. The radioactivity readings of the atmosphere were taken periodically in order to check the amount of radioactivity absorbed by the plants. The second and third sets of bean plants were exposed to $C^{14}O_2$ in a similar manner, three days and seven days, respectively, after the treatment with 2,4-D. The amounts of $BaC^{14}O_3$ used for these three experiments were 4.3 mg, 6.3 mg, and 5.1 mg respectively (20.6 μc/mg).

After exposure to radioactive CO_2, the entire plants were harvested and dried overnight at 55° C in a vacuum oven. The samples were then weighed, ground and stored in an evacuated desiccator.

RADIOACTIVITY MEASUREMENTS: Weighed aliquots of each sample were oxidized by dry combustion (2). The carbon dioxide was quantitatively absorbed in carbonate-free sodium hydroxide solution and precipitated as barium carbonate. The radioactivity of the barium carbonate samples was measured with a thin mica window Geiger-Müller counter (1.9 mg/cm^2) attached to a scaler in the usual manner. All measurements were corrected to zero thickness.

After the paper chromatograms were developed (see Isolation and Analyses) and dried, the radioactivity of the amino acids was determined. The paper strips were counted at centimeter intervals through a one centimeter slit. The Geiger counter was placed as close as possible to the strip to increase the counting efficiency. Several one centimeter sections of the paper chromatograms containing various amounts of radioactivity were oxidized, and the radioactivity was redetermined as barium carbonate. These comparison values were plotted to determine a correction curve. Thus the radioactivity found in the paper chromatograms could be expressed as $BaCO_3$ activity.

ISOLATION AND ANALYSES: Weighed amounts of each sample (100 mg) were autoclaved with 10 ml of 2 *N* HCl in sealed tubes for 10 hrs at 15 lbs pres-

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[2] Presented before the American Society of Plant Physiologists at the 35th meeting of the American Association for the Advancement of Science, Pacific Division, Pullman, Washington. The data in this paper was taken from a thesis presented by T. J. Akers to the faculty of the Graduate School of Oregon State College in partial fulfillment of the requirements for the degree of Master of Science in Chemistry. This work was supported by a grant from the Atomic Energy Commission.

[3] Technical Paper No. 926, Oregon Agricultural Experiment Station.

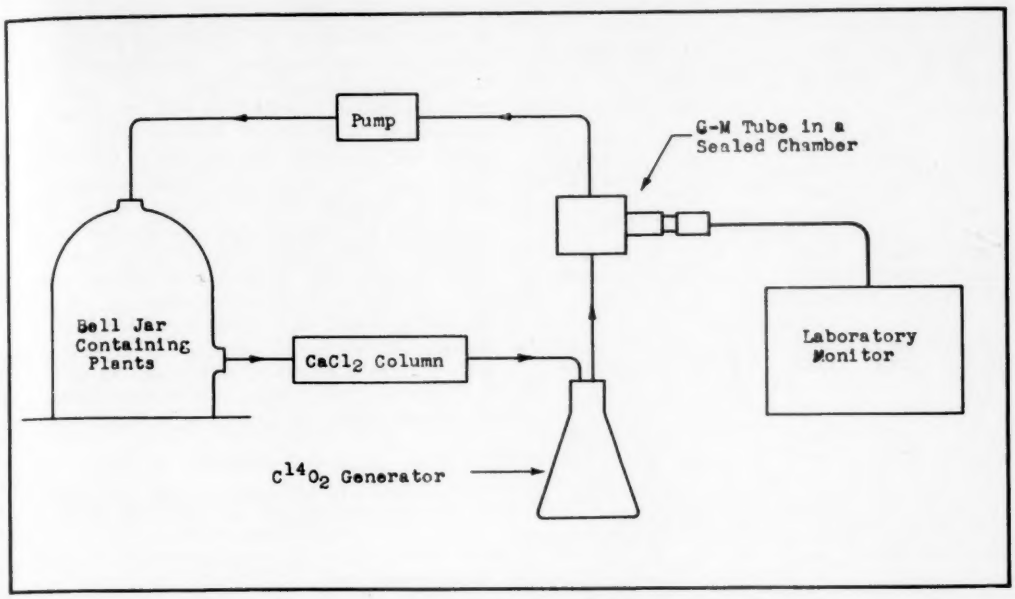

FIG. 1. Block diagram of the sealed system used for $C^{14}O_2$ absorption.

sure. The excess hydrochloric acid was removed from the hydrolysate by repeated addition of water and evaporation to dryness in vacuo. The hydrolysate was made up to 100 ml and the residue was removed by filtering through a dry filter.

The acidic compounds were separated from the neutral and basic compounds by means of ion-exchange columns of Amberlite IR-4B as follows: Several glass columns, 16 mm in diameter, were filled to a height of 25 cm with resin in the basic form. The columns were back washed, charged with 4 % NH_4-OH, and washed with distilled water until neutral.

The corresponding pairs of treated and control samples were run simultaneously to reduce the errors due to differences in operation. An aliquot of each solution was allowed to pass through a resin column at the rate of approximately 0.5 ml per minute. When the last of the solution reached the top of the resin bed, the column was washed with distilled water, allowing no break in the continuity of the liquid phase. This washing was continued until no activity was detected in the effluent. An excess amount of 4 % HCl was passed through the column to elute the acidic compounds (including aspartic and glutamic acids) from the resin. The HCl was again removed from the effluent by the method described previously. The samples were then diluted to 25 ml.

The aspartic and glutamic acid were separated by means of paper chromatography. Aliquots of the solutions were applied quantitatively as spots or lines to strips of Whatman #1 filter paper (1″ × 22″). These strips were developed in aqueous phenol for 18 hours by the descending front method and were dried at room temperature after developing.

After the radioactivity of the unknown strips had been measured, quantitative determination of the amino acids present was made by Block's method (1) using ninhydrin as a color reagent. The relative color density was measured with a Photovolt Electronic Densitometer, model 525, using a blue filter. The results were compared with a standard curve prepared in the similar manner from known amounts of amino acids. If carefully conducted, accuracies of ± 5 % are readily obtained by this method.

RESULTS AND DISCUSSION

Approximately 90 % of the radioactivity was absorbed by the bean plants in the first 8 hours of exposure. The amount of C^{14} incorporated into each plant sample is given in table I. It is interesting to note that the 2,4-D treated plants absorbed much less radioactive CO_2 than did the control plants. Furthermore, this difference in absorption which is shown in table I as the ratio of total radioactivity, is relatively the same for all three experiments. (T/C = 0.615, 0.622 and 0.615 for 1st, 3rd and 7th day samples respectively.) These results, which indicate that 2,4-D treatment reduces the rate of photosynthesis, agree closely with the work reported by Freeland (3).

The results of the chemical analyses are given in table II. The total aspartic acid and glutamic acid content of each plant sample is shown on the left side of the table. Since the number of plants in each experiment was small and the weights of the plant varied somewhat, the percentage of amino acids in each sample was calculated to obtain comparable values. These data are shown on the right side of table II.

In the first-day samples, the percentages of both

TABLE I

EFFECT OF 2,4-D TREATMENT ON PHOTOSYNTHESIS IN BEAN PLANTS AS DETERMINED BY ANALYSES OF C^{14} IN PLANT TISSUE

SAMPLE	WT OF PLANT SAMPLE, MG	TOTAL RADIOACTIVITY, CPM × 10^3 **	$BaC^{14}O_3$ USED, MG *	SPECIFIC ACTIVITY, CPM × 10^3/MG
		1st Day		
Treated	864.6	3,817		4.41
Control	688.1	6,203	4.3	9.01
T/C		0.615		
		3rd Day		
Treated	893.4	6,419		7.18
Control	879.4	10,312	6.3	11.73
T/C		0.622		
		7th Day		
Treated	876.3	4,929		5.62
Control	1079.9	8,010	5.1	7.42
T/C		0.615		

* Specific activity of $BaC^{14}O_3$ is 20.6 μc/mg.
** The activity was measured by a thin mica window G-M counter (1.9 mg/cm²).

amino acids in whole plants were essentially the same in both control and treated plants, while in the 3rd and 7th day samples a great decrease in these amino acids was noted in the 2,4-D treated plants.

Sell et al (7) and Weller et al (9) have reported the effect of 2,4-D treatment on changes in chemical composition of the leaves, stems and roots of the red kidney bean plants. From their more complete results on the analyses of eleven amino acids, they have suggested that 2,4-D treatment causes a change in the character of the plant protein. These findings of the change in aspartic acid and glutamic acid concentration agree closely with their results.

The results of the radioactive analyses are summarized in table III. On the left side of the table, the total radioactivity of aspartic and glutamic acids in each plant sample is shown. Since different amounts of radioactive barium carbonate were used in the three experiments and since the rate of fixation of the radioactivity was greatly reduced in the treated plants, the percentage incorporation of C^{14} into each amino acid was calculated. These percentages, as shown on the right side of the table, indicate that the amounts of C^{14} incorporated into aspartic and glutamic acids were approximately 3 to 4 times greater in the treated than in the control plants, with the most pronounced effect on the third day. It is also interesting to note that the incorporation of C^{14} into glutamic acid increased with the age of bean plants while a decrease was found in the aspartic acid.

If the metabolic cycling of C^{14} was unaffected in the treated plants, the amount of radioactivity incorporated into the amino acids should indicate the difference in the rate of synthesis during the experimental period. Since 2,4-D treatment increased the incorporation of C^{14} into aspartic and glutamic acids, it is logical to conclude that the rate of synthesis of these two amino acids had been increased. However, the total quantity of these two amino acids was markedly decreased in the treated plants particularly in the third and the seventh day samples. Therefore, 2,4-D treatment must increase the rate of oxidation or catabolism of these amino acids, and this increase must exceed the increase in the rate of synthesis. Rebstock et al (6) and Freiburg (4) found that 2,4-D caused increases in proteinase and polypeptidase activities which lends some support to these findings.

Carbohydrate and sugar reserves in bean plants are readily depleted following 2,4-D treatment, as reported by several workers (7, 8, 9) and the synthesis of aspartic and glutamic acids is increased as indi-

TABLE II

EFFECT OF 2,4-D TREATMENT ON THE AMOUNTS OF ASPARTIC AND GLUTAMIC ACIDS IN BEAN PLANTS

TOTAL AMOUNT IN PLANT TISSUE (μGM)				% AMINO ACID IN PLANT TISSUE			
ASPARTIC ACID		GLUTAMIC ACID		ASPARTIC ACID		GLUTAMIC ACID	
TREATED	CONTROL	TREATED	CONTROL	TREATED	CONTROL	TREATED	CONTROL
			1st Day				
3644	3016	2114	1569	0.421	0.438	0.245	0.228
			3rd Day				
3858	4431	1336	2351	0.432	0.504	0.150	0.267
			7th Day				
2178	4117	1234	2952	0.244	0.381	0.141	0.273

All values are averages of triplicate determination.

TABLE III

EFFECT OF 2,4-D TREATMENT ON THE INCORPORATION OF C^{14} INTO ASPARTIC AND GLUTAMIC ACIDS IN BEAN PLANTS

TOTAL RADIOACTIVITY, CPM × 10^3				% INCORPORATION OF C^{14}					
ASPARTIC ACID		GLUTAMIC ACID		ASPARTIC ACID			GLUTAMIC ACID		
TREATED	CONTROL	TREATED	CONTROL	TREATED	CONTROL	T/C	TREATED	CONTROL	T/C
				1st Day					
26.9	16.0	32.5	16.7	0.71	0.26	2.73	0.85	0.27	3.15
				3rd Day					
57.3	22.1	113.8	51.0	0.89	0.21	4.15	1.77	0.50	3.58
				7th Day					
13.4	6.4	91.3	57.3	0.27	0.08	3.40	1.85	0.72	2.59

All values are averages of triplicate determination.

cated by this work. It would seem, therefore, that during photosynthesis the ordinary reaction from C_3 to C_6 sugar is greatly inhibited, more carbon dioxide enters the Kreb's cycle through the condensation of C_3 to C_4 units, which subsequently increases the synthesis of amino acids. This increase in the synthesis of aspartic and glutamic acids might probably result from the normal process of transamination, and the decrease in total amounts of these amino acids during this seven day period could then be solely due to an increased catabolism of the protein. The carbon fragments from this oxidation might not return for resynthesis of amino acids as suggested by Payne et al (5) in her experiment with potato tubers. In view of the fact that the amount of aspartic and glutamic acid, measured by this method, included both free and bonded amino acids as well as from the hydrolysis of asparagine and glutamine, it is impossible at present to determine whether the primary effect of 2,4-D treatment is on the free amino acids or on the amides. However, this result undoubtedly indicated that 2,4-D treatment may affect both the synthesis and the oxidation of these two amino acids in bean plants.

Summary

1. The rate of photosynthesis in bean plants was markedly reduced by 2,4-D treatment. The rate of reduction remained essentially constant over the seven day period after treatment.

2. The incorporation of C^{14} into aspartic and glutamic acids was 3 to 4 times greater in the treated plants than in the control plants. The most pronounced effect was observed in plants harvested on the third day.

3. Total amounts of aspartic acid and glutamic acid decreased in the treated plants, especially in the plants harvested on the seventh day after treatment. A possible explanation for the increased incorporation of C^{14} and more rapid loss of aspartic and glutamic acids is discussed.

Literature Cited

1. Block, R. J. Estimation of amino acids on paper chromatograms. Anal. Chem. 22: 1327–1332. 1950.
2. Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M., and Yankwich, P. F. Isotopic Carbon. Pp. 82–88. John Wiley & Sons, New York. 1949.
3. Freeland, R. O. Effects of growth substances on photosynthesis. Plant Physiol. 24: 621–628. 1949.
4. Freiberg, S. R. Effects of an exogenous growth regulator on proteolytic enzymes of the soybean plant. Science 115: 674–675. 1952.
5. Payne, M. C., Fults, J. L., and Hay, R. V. The effect of 2,4-D treatment on free amino acids in potato tubers. Amer. Potato Jour. 29: 142–150. 1952.
6. Rebstock, T. L., Hamner, C. L., Ball, C. D., and Sell, H. M. Effect of 2,4-dichlorophenoxyacetic acid on proteolytic activity on red kidney bean plants. Plant Physiol. 27: 639–643. 1952.
7. Sell, H. M., Leucke, R. W., Taylor, B. M., and Hamner, C. L. Changes in chemical composition at the stems of red kidney bean plants treated with 2,4-dichlorophenoxyacetic acid. Plant Physiol. 24: 295–299. 1949.
8. Smith, F. G. The effect of 2,4-dichlorophenoxyacetic acid on the respiratory metabolism of bean stem tissue. Plant Physiol. 23: 70–83. 1948.
9. Weller, L. E., Leucke, R. W., Hamner, C. L., and Sell, H. M. Changes in chemical composition of leaves and roots of red kidney bean plants treated with 2,4-dichlorophenoxyacetic acid. Plant Physiol. 25: 289–293. 1950.

PARTICULATE ADENYLIC KINASE IN HIGHER PLANTS[1,2]

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Adenylic kinase (AK) which catalyzes the reversible reaction

$$(1) \qquad 2\text{ADP} \rightleftharpoons \text{ATP} + \text{AMP}$$

was first described by Colowick and Kalckar (1) and named by them myokinase. The name was assigned because rabbit and frog skeletal muscle were the richest sources of the enzyme. Kotelnikova (2) has since shown that the enzyme is widely distributed in the animal organism, being present in liver, kidney, and erythrocytes in addition to muscle. The enzyme has been shown to be associated with the mitochondria in rat and mouse liver preparations (3, 4, 5).

As far as the plant kingdom is concerned, the enzyme has been described in yeast (6) but no reference is to be found in the literature concerning higher plants. However, Loomis (7), in his doctoral dissertation, and Stumpf (31) in a review present evidence for the presence of the enzyme in extracts of acetone powders of pumpkin seedlings and manroot leaves and also mention a personal communication from Axelrod, Bandurski and Campbell stating they had shown adenylic kinase (AK) activity in plant tissue. The present study demonstrates the presence of an adenylic kinase associated with the mitochondria of cotyledons from peanut and lupine seedlings and the chloroplasts from spinach and tobacco leaves. Some

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properties of the mitochondrial and chloroplast enzymes have also been described.

Materials and Methods

Peanuts (*Arachis hypogea* L. var. Virginia Jumbo) and lupine (*Lupinus albus* L.) were germinated in flats of moist peat moss or vermiculite. The lupine were germinated in the dark. The peanuts were treated with fungicide as described by Stumpf (8). When 5 to 8 days old the plants were harvested and the cotyledons removed and washed and the mitochondria isolated as described previously (8). Lyophilized mitochondria were prepared as described by Mazelis and Stumpf (9). Spinach and tobacco were grown in nutrient solution (10). When 4 to 6 weeks old the leaves were harvested, washed in distilled water, and stored in pliofilm bags at 10° C until used. The leaves were used within one week of harvest. The leaf blade, after separation from the midrib, was treated in the following manner: 80 to 100 gm lots of leaf laminae were chopped with a knife into small pieces and triturated in a Warburg blendor at 4° C in 150 ml of 0.2 M TRIS (Tris-(hydroxymethyl)-aminomethane) buffer at pH 7.4 which was 0.5 M with respect to sucrose. The blending was carried out at 80 volts for 45 to 60 seconds. The homogenate was filtered through two layers of cheesecloth and the filtrate centrifuged at $600 \times g$ for one minute. The sediment was discarded. The supernatant fluid was centrifuged for 30 minutes at $2000 \times g$. The supernatant from this centrifugation was discarded and the sediment resuspended in TRIS-sucrose. The resuspended material was centrifuged at $1300 \times g$ for 10 minutes. If whole chloroplast preparations were desired, the sediment from this centrifugation was resuspended in TRIS-sucrose solution for use as the enzyme source, otherwise the sediment was resuspended in cold distilled water, which had been de-ionized by passage through a Barnstead Bantam demineralizer, and the suspension centrifuged for 10 minutes at $1300 \times g$. The pellet obtained was again resuspended in cold, de-ionized, distilled water and lyophilized. All centrifugations were carried out in an International Refrigerated Centrifuge at 0° C.

Semi-quantitative estimates of adenylic kinase activity were obtained by incubating the enzyme preparation with ADP^{32} at room temperature. After 30 minutes the reaction was stopped with 1.5 ml of 1.5 M $HClO_4$ and the resulting precipitate removed by centrifugation. The excess perchlorate ion was removed by neutralizing with KOH and freezing overnight (5). The precipitate obtained was centrifuged off. Known aliquots of the supernatant were spotted on Whatman No. 1 filter paper and an ascending chromatogram run in the two-phase solvent system of Cohn and Carter (12) with known adenylic acid (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP) standards. After 24 hours the chromatogram was removed and dried and a radioautograph made. The total number of counts per minute (cpm) spotted on paper was determined by placing the same volume aliquot on a stainless steel planchet and counting with a standard Geiger-Müller counter. The radioactive areas were cut from the paper and eluted, transferred to steel planchets and counted.

ADP^{32} was synthesized by use of lupine mitochondria prepared by the method of Humphreys and Conn (11) with inorganic P^{32} in a phosphorylation medium similar to that of Kielley and Kielley (5). The ADP^{32} was isolated by ion-exchange chromatography (12).

Spectrophotometric assays of AK activity were made by use of a slight modification of the hexokinase–glucose-6-phosphate dehydrogenase system of Kornberg (13).

Nitrogen determinations were done by digestion of the sample followed by direct Nesslerization of the digested sample.

AMP, ADP, and ATP were the sodium salts purchased from the Pabst Laboratories. Hexokinase was generously supplied by Dr. Robert Bartsch. Glucose-6-phosphate dehydrogenase was prepared by the method of Kornberg (14). Triphosphopyridine nucleotide (TPN) was the sodium salt obtained from Pabst Laboratories.

Results

Mitochondrial preparations of lupine and peanut cotyledons and whole chloroplast preparations from spinach and tobacco leaves possess adenylic kinase activity which is stable to freezing. Mitochondrial suspensions retain their activity for 3 months at $-10°$ C and frozen chloroplast suspensions retain their activity for over a month at the same temperature. Some preparations were lyophilized and the lyophilized powders retained their activity for an indefinite period.

The enzyme was easily solubilized from the lyophilized mitochondria and chloroplasts. One percent suspensions (10 mg/ml) of the mitochondrial powders were extracted for approximately one hour with cold distilled de-ionized water. Three percent suspensions of the lyophilized chloroplast preparations were suspended and extracted in the same way. The extracts were clarified by centrifugation at 0° C. The peanut extract was centrifuged in a Spinco ultracentrifuge at $100{,}000 \times g$ for 15 minutes. The lupine and chloroplast extracts were clarified by centrifugation in an International Refrigerated Centrifuge at $16{,}000 \times g$ for 20 minutes.

Aliquots of the clear enzyme extract were assayed for N as described above. Under the extractive conditions described the clear lupine mitochondrial extract (ME) and spinach chloroplast extract (CE) contained 0.20 mg N per ml of extract.

pH Optimum of Reaction: The adenylic kinase activity of the spinach CE and peanut ME was examined for a pH optimum over the pH range 5.8 to 8.7. Phosphate buffers were used from pH 5.8 to 8.0 and TRIS buffers from 7.4 to 8.7. The buffers were duplicated at pH 7.4 and pH 8.0 in order to deter-

TABLE I

ACTIVATION OF ADENYLIC KINASE BY METAL CATIONS

	SYSTEM	TOTAL CPM ON PAPER	TOTAL CPM RECOVERED	% TOTAL CPM RECOVERED AS ADP	% TOTAL CPM RECOVERED AS ATP
	Peanut				
a	Control	2370	2005	100	..
b	" + Mg^{++}	2875	2620	45	55
c	" + Mn^{++}	2610	2410	73	27
d	" + Co^{++}	1805	1645	66	34
e	" + Ca^{++}	1945	1920	75	25
f	" + Zn^{++}	1200	1500	100	..
	Lupine				
a		2310	2030	100	..
b		1815	1955	45	55
c		1155	1165	66	34
d		1800	1785	70	30
e		1820	1705	83	17
f		1625	1450	100	..
	Spinach				
a		1665	1565	90	10
b		1845	1760	55	45
c		1530	1425	73	27
d		1500	1605	79	21
e		2120	2025	90	10
f		1565	1320	94	6

Control system: *Peanut and lupine:* 0.5 ml EDTA-treated, dialysed ME, 0.2 ml 2 M TRIS, pH 7.4, 3 μM ADP containing 10^6 cpm as ADP^{32}. Final volume 2 ml. *Spinach:* 0.5 ml dialysed CE, 0.2 ml 2 M TRIS, pH 7.4, 3 μM ADP containing 10^6 cpm ADP^{32}. Final volume 2 ml.

Added 15 μM of the desired metal to determine the activation. Reaction stopped and products chromatographed as described in the section on "Methods."

mine if there was an effect of the buffer not due to pH. Since the AK activity was independent of pH over the range examined, pH 7.4 was used as a standard pH for all the following investigations.

METAL ACTIVATION: Six- and 24-hour dialysis of the peanut and lupine ME against cold de-ionized water had no effect on the enzyme. Preincubation of the ME with 10 μM of ethylenediaminetetraacetate (EDTA) per ml of ME for a short time completely abolished AK activity. This implied the necessity of some metal for enzyme activity. The EDTA treated extract was then dialyzed for 6 hours in the cold against distilled water. This was done to preclude the possibility that on the addition of various cations they would displace the natural metal activator from the EDTA complex and thus reactivate the enzyme. Dialysis of the EDTA-treated extract yielded an inactive preparation which could be reactivated by Mg^{++} and to a lesser extent by Mn^{++}, Co^{++}, and Ca^{++}. Zn^{++} had no effect (table I).

The CE did not require pre-incubation with EDTA to demonstrate a metal requirement. There was a small AK activity if no metal was added, but 3-hour dialysis removed this small residual activity almost completely. Full activity could be restored by addition of Mg^{++}. There was a lesser activation on addition of Mn^{++} or Co^{++} while Ca^{++} and Zn^{++} had no effect. The results are summarized in table I. It appears as if the metal is more tightly bound to the protein in the case of the mitochondrial enzyme. The metal-protein bond in the chloroplast enzyme is more easily broken. The chloroplast enzyme appears to be irreversibly inactivated by 24-hour dialysis.

INHIBITION STUDIES: Attempts to inhibit the AK of spinach CE and the lupine and peanut ME were made using well-known metabolic inhibitors. The results, shown in table II, clearly indicate that there was no inhibition of the mitochondrial AK at the levels of inhibitor concentrations employed. Since there appeared to be some slight effect of fluoride on the spinach CE enzyme the inhibitory effect of a series of fluoride concentrations on the spinach AK and lupine AK was tested. The results (table III) demonstrated that there is an increasing inhibition of the chloroplast enzyme with increasing concentrations of F^- until a final F^- concentration of 0.02 M is reached. Increasing the F^- concentration past this

TABLE II

INHIBITION STUDIES OF MITOCHONDRIAL AND CHLOROPLAST ADENYLIC KINASE

SYSTEM	LUPINE				SPINACH			
	TOTAL CPM ON PAPER	TOTAL CPM RECOVERED	% TOTAL CPM RECOVERED AS ADP	% TOTAL CPM RECOVERED AS ATP	TOTAL CPM ON PAPER	TOTAL CPM RECOVERED	% TOTAL CPM RECOVERED AS ADP	% TOTAL CPM RECOVERED AS ATP
Control	2250	1565	44	56	2115	1850	45	55
+ F^- (10^{-2} M)	1955	1755	58	42	1890	1860	75	25
+ azide (10^{-2} M)	1955	2130	42	58	2215	1700	57	43
+ arsenate (10^{-2} M)	2250	2005	49	51	1940	1470	51	49
+ iodoacetamide (IAA) (10^{-3} M)	1775	1770	41	59	2050	1750	53	47
+ *p*-mercurichlorobenzoate (PCMB) (10^{-3} M)	2395	2525	51	49	2280	1550	64	36

The final concentrations of the inhibitor used are in parentheses.

Control system: 0.5 ml ME or CE, 15 μM $MgCl_2$ 3 μM ADP containing 10^6 cpm ADP^{32}, 0.2 ml 2 M TRIS, pH 7.4. Total volume 2 ml.

TABLE III

INHIBITION OF ADENYLIC KINASE BY INCREASING F^- CONCENTRATION

FINAL F^- CONC M	LUPINE				SPINACH			
	TOTAL CPM ON PAPER	TOTAL CPM RECOVERED	% TOTAL CPM RECOVERED AS		TOTAL CPM ON PAPER	TOTAL CPM RECOVERED	% TOTAL CPM RECOVERED AS	
			ADP	ATP			ADP	ATP
0	1650	1690	42	58	1910	1770	52	48
2.5×10^{-3}	1665	1735	45	55	1870	1835	59	41
5×10^{-3}	1660	1895	41	59	1720	1465	60	40
1×10^{-2}	1625	1755	53	47	1455	1520	69	31
2×10^{-2}	1670	1775	63	37	1965	1820	79	21
3×10^{-2}	1780	1690	67	33	1805	1875	82	18

Reaction system same as that for table II.

point had relatively little effect. The maximum inhibition obtained was 60 % of the activity of the control. There was no great effect of F^- on the lupine mitochondrial enzyme until 0.02 M F^- is reached. At this concentration a 40 % inhibition was observed. The inhibition of the mitochondrial enzyme by F^- appears to fall in an intermediate region as compared to the results with liver mitochondrial adenylic kinase. Barkulis and Lehninger (3) found that 0.03 M F^- was sufficient to abolish the AK activity of washed rat liver mitochondria, while a 0.01 M concentration had no effect. On the other hand Siekevitz and Potter (15) observed that fluoride inhibited rat liver mitochondrial AK at a concentration of 0.01 M. The liver mitochondrial enzyme was found to be easily solubilized either by blending (3) or by sonic disintegration of the particles (5). It is interesting to note that a soluble adenylic kinase from liver homogenates has been described which is only weakly inhibited by 0.03 M F^- (16). Thus the rather indeterminate inhibition of F^- of the adenylic kinase of the spinach CE and lupine ME appears analogous to the results obtained with the enzyme from rat liver.

HEAT STABILITY OF MITOCHONDRIAL AND CHLOROPLAST ENZYMES: One of the most interesting features of the adenylic kinase from muscle is the fact that heating the enzyme for 10 minutes at 100° C in 0.1 *N* HCl only causes an inactivation of about 30 % (1). The picture is somewhat confused as far as the stability of the enzyme in rat liver is concerned. In this tissue the particulate enzyme is unstable to the heat-acid treatment (3, 4), although the soluble enzyme of Leuthardt and Bruttin (16) retained its activity after this drastic treatment. This raised the question as to whether there were two enzymes in liver or whether the properties changed on solubilization from the mitochondria. The yeast adenylic kinase has been shown to be relatively stable to the heat-acid treatment, losing only 50 % of its activity (6).

A unit of enzyme activity will be defined as that amount which will form enough ATP to react with the glucose-6-phosphate dehydrogenase–hexokinase system so as to cause an increase of optical density of 0.001 per minute at 340 $m\mu$ by the reduction of TPN. With the period of 4 to 8 minutes on the graphs of figures 1 to 3 as a period when the TPN reduction is proceeding linearly, table V gives the results of the amount of inactivation by the acid-heat treatment. The amount of inactivation of the mitochondrial enzymes are of the same order of magnitude as the relatively stable yeast (6) and muscle (1) enzymes. As is shown in figures 1 and 2, the soluble enzyme from the mitochondria retained most of its activity after treatment with heat and acid. The soluble chloroplast enzyme, however, loses the major portion of its activity (fig 3). The chloroplast AK has the interesting property of retaining its activity after this acid-heat treatment if it is not separated from the insoluble matrix by water extraction. As seen in figure 3 and also in table IV, the solubilized chloroplast enzyme loses its activity almost completely, but if the whole suspension is used the enzyme maintains its activity to a great extent after the heat-acid treatment.

When fresh or lyophilized mitochondrial suspensions or fresh chloroplast preparations were incubated with ADP^{32}, radioactive spots showed up corresponding to ATP, ADP, and inorganic P. Heating the mitochondrial preparations in 0.1 *N* HCl for 10 minutes at 100° C abolished the inorganic P spot. Incubating with high concentrations of unlabeled AMP decreased the intensities of the radioactive ATP and inorganic P areas on the paper chromatogram. When the suspension of lyophilized mitochondria was centrifuged and the clear supernatant used as the enzyme source, only ADP and ATP were labeled. These results imply that an ATPase is associated with the mitochondria and is not solubilized by water extraction as is the adenylic kinase. A similar insoluble ATPase associated with the mitochondrial fraction has been observed in mouse liver (17). The fresh chloroplast preparations also have an ATPase but the activity of the enzyme appears to decrease on storage at – 10° C. After several days, the ATPase activity has decreased to a large degree as determined by the amount of labeling in the inorganic P spot on a radioautograph.

CHOLEATE TREATMENT OF LYOPHILIZED CHLOROPLASTS: The question arises whether there are two

enzymes in the chloroplast, an easily extracted heat-labile one and an insoluble heat-stable one, or whether the properties of the enzyme are changed in some way on going into solution and being separated from the insoluble portion.

A 3% suspension of lyophilized spinach chloroplasts was made up and, after extraction and centrifugation, the clear CE was decanted. The residue

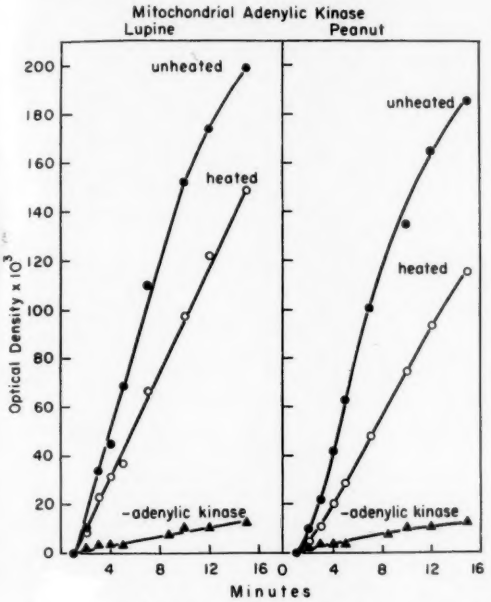

FIG. 1 (*left*). Activity of adenylic kinase of lupine mitochondria after heating 10 minutes at 100° C in 0.1 *N* HCl. Cuvette contained 0.2 ml of ME preparation, 0.1 ml of 0.1 M glucose, 0.1 ml of 2 M TRIS, pH 7.4, 0.1 ml of 0.1 M $MgCl_2$, 0.1 ml of a glucose-6-phosphate dehydrogenase solution containing 1 mg per ml of the lyophilized preparation, 0.1 ml of a hexokinase solution containing 1 mg of enzyme preparation per ml in a 1% glucose solution, 0.1 ml of TPN solution containing 100 γ of TPN, and 0.1 ml of 0.01 M ADP. Add water to make up to 3 ml final volume. Control cuvette had no TPN. Start reaction by adding ADP. Measure increase of optical density at 340 mμ.

FIG. 2 (*right*). Activity of peanut mitochondrial adenylic kinase under same conditions.

was re-extracted two more times with water and re-centrifuged, discarding the supernatant each time. The washed residue was then re-extracted with water and after centrifugation the supernatant (S_4) saved and the residue again resuspended in water. A comparison of the AK activity of the CE, S_4, and residue was made with labeled ADP. Also the effect of acid-heat treatment on each fraction was examined. An active adenylic kinase was present in the CE and washed residue. There was no activity in the S_4 fraction. Heat-acid treatment of the CE gave the

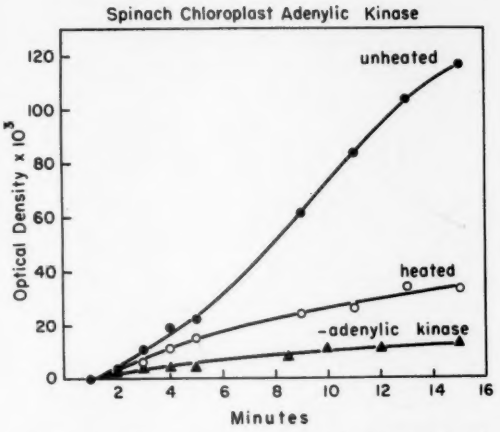

FIG. 3. Inactivation of spinach chloroplast adenylic kinase by heat-acid treatment. 0.2 ml of CE preparations in cuvette. Other reactants same as for figure 1 above.

usual large inactivation, but the same treatment on the residue had no effect. There apparently is a portion of the AK activity which cannot be extracted by water from the lyophilized chloroplast.

The washed residue above was treated with 10 mg sodium choleate per ml of suspension. After one hour of such treatment the suspension was clarified by centrifugation and the clear supernatant decanted. The sediment was resuspended in water and a comparison of the AK activity and the effect of heat-acid treatment on the clear supernatant and the choleate-treated residue was made. The choleate treatment solubilized the remaining AK in the washed residue. Only a small amount of activity remained in the insoluble portion. The clear supernatant from this

TABLE IV

HEAT STABILITY OF CHLOROPLAST ADENYLIC KINASE

ENZYME PREPARATION		TOTAL CPM ON PAPER	TOTAL CPM RECOVERED	% TOTAL CPM RECOVERED AS ADP	% TOTAL CPM RECOVERED AS ATP
Tobacco	CL*	2500	2050	50	50
"	CE	2655	2560	68	32
Heat-Acid treated	CL	2390	2000	45	55
" " "	CE	1075	1020	86	14
Spinach	CL	970	755	54	46
"	CE	1005	795	53	47
Heat-Acid treated	CL	915	870	63	37
" " "	CE	970	905	83	17

* CL is a 3% suspension of lyophilized chloroplasts; CE is chloroplast extract.

Reaction mixture: 0.5 ml enzyme preparation, 15 μM $MgCl_2$ 3 μM ADP containing 10^6 cpm ADP^{32}, 0.2 ml TRIS, pH 7.4. Final volume 2 ml. After heating in boiling H_2O bath for 10 minutes in 0.1 *N* HCl the heat-acid treated enzyme preparation is neutralized with KOH prior to use.

TABLE V

ENZYME INACTIVATION BY TREATMENT WITH 0.1 *N* HCl AT 100° C

ENZYME SOURCE	TREATMENT	UNITS OF ACTIVITY (CORRECTED FOR BLANK)	% INACTIVATION
Lupine mitochondria	Unheated	17.0	..
" "	Heated	10.0	41
Peanut "	Unheated	16.0	..
" "	Heated	8.0	50
Spinach chloroplasts	Unheated	6.5	..
" "	Heated	1.0	85

treatment had good activity which, however, was considerably decreased by the heat-acid treatment.

PROOF OF THE PROTEIN NATURE OF THE ADENYLIC KINASE ACTIVITY: Since the AK from lyophilized mitochondrial preparations was stable to such drastic treatment as heating in acid solution, the question was raised whether there was a protein associated with this activity. Studies were made with peanut ME to answer this question. Peanut ME which had been frozen 31 days was thawed. An aliquot was incubated for one hour with 0.2 ml of a papain solution (0.81 units of activity/mg) and 0.1 ml of a neutralized glutathione solution (2.5 mg/ml) to activate the papain. Another aliquot of ME was incubated with 0.2 ml of the papain-glutathione solution which had been heated previously for 10 minutes in a boiling water bath. After one hour the two enzyme preparations were incubated with ADP^{32} as usual in order to determine AK activity. A control was run with ME alone. After radioautography of the chromatogram of the reaction products, it was seen that the ME which had been treated with heated papain had an active adenylic kinase. However, the papain-treated ME had no AK activity. Similar results were obtained upon incubating heat-acid treated ME with active and heat-inactivated papain.

DISCUSSION

The apparent concentration of adenylic kinase in the chloroplasts of spinach and tobacco leaves is of interest for several reasons. First, this is one of the few enzymes which has been specifically identified in chloroplasts. Li and Bonner (19) identified a polyphenol oxidase localized in tea leaf chloroplasts and the evidence for chloroplast localization of phosphorylase (20) and catalase (21) is fairly substantial. The whole complex of enzymes involved in the Hill reaction (18) is known to be present in the chloroplast but the individual enzymes have not been identified or characterized. In addition, the presence of AK is of interest from a consideration of how the chloroplast is involved in changing light energy to chemical energy. Arnon, Whatley, and Allen (22) have recently presented evidence indicating that under the action of light, isolated chloroplasts can incorporate inorganic phosphate into ATP using AMP as an acceptor. ADP was found to be as effective as AMP. With adenylic kinase present, it would seem reasonable to assume that the primary phosphate acceptor is ADP and that AMP acts by the presence of small contaminating amounts of ADP or ATP in the added AMP or the chloroplast preparation itself. Thus, adenylic kinase would insure a means of generating ADP in the presence of any two of the components involved in reaction (*1*).

The interesting observation that the chloroplast adenylic kinase is relatively stable to an acid-heat treatment while still attached to the insoluble chloroplast structure, but on going into solution is inactivated by this same treatment, implies either the chloroplast structure somehow stabilizes the enzyme or that the properties of the enzyme change on going into solution. The properties of the soluble liver enzyme (16) compared to the liver mitochondrial enzyme (3) appear analogous. In this case, however, solubilization of the enzyme stabilized the enzyme to the acid-heat treatment but the enzyme still attached to the mitochondrion was unstable.

Speculation on the role of adenylic kinase in cell metabolism leads to a belief that this enzyme occupies an important position in the interchange of high-energy phosphate in nucleotide-involved reactions. Strominger, Heppel, and Maxwell (23) have described a nucleoside monophosphokinase which will transfer the terminal phosphate from a nucleoside triphosphate to a nucleoside monophosphate, provided one of the components is an adenine nucleotide. Berg and Joklik (24) have demonstrated an enzyme, nucleoside diphosphokinase, which will transfer phosphate from a nucleoside triphosphate to a nucleoside diphosphate. Again an adenine nucleotide appears to be a necessary component. In recent years, inosine diphosphate has been shown to be the phosphate acceptor in the phosphoenolpyruvate-CO_2 condensation to oxalacetate (25); the uridine nucleotides are involved in carbohydrate transformations (26, 27, 28), cytidine nucleotides in phospholipid synthesis (29), and guanosine nucleotides as cofactors for the esterification of inorganic phosphate in α-ketoglutarate oxidation (30). The maintenance of an adequate balance between the three adenine nuclotides would seem to be related, therefore, not only to providing a substrate for the esterification of inorganic phosphate into high-energy linkage, but also to be important in regulating many other metabolic pathways not directly involving adenine nuclotides but dependent on other nucleotides.

SUMMARY

1. An adenylic kinase has been found associated with lupine and peanut cotyledon mitochondria and also with whole chloroplasts isolated from spinach and tobacco leaves.

2. The mitochondrial enzyme requires treatment with ethylenediaminetetraacetate to demonstrate a metal requirement. Mg^{++} is most effective, Mn^{++}, Co^{++} and Ca^{++} activate to a lesser extent. The enzyme is unaffected by F^- until a concentration of 0.02 M is

reached at which point the enzyme is inhibited about 40 %. There is a 50 % loss of activity on heating for 10 minutes at 100° C in 0.1 *N* HCl.

3. The chloroplast enzyme does not retain the metal as firmly as the mitochondrial adenylic kinase. Mg^{++} is most effective as an activator. Mn^{++} and Co^{++} activate to a lesser degree. Increasing F^- concentrations cause increasing inhibition until 0.02 M F^- is reached, at which point the enzyme is approximately 60 % inhibited. Increasing F^- concentrations above this point does not significantly increase the amount of inhibition.

4. The chloroplast enzyme, when solubilized, is unstable to heat-acid treatment. However, when still associated with the chloroplast structure, the enzyme retains a great deal of its activity.

5. The possible role the enzyme fulfills in the process of converting light to chemical energy in photosynthesis, and its function in a broad sense in cell metabolism is discussed.

Literature Cited

1. Colowick, S. P. and Kalckar, H. M. The role of myokinase in transphosphorylations. I. The enzymatic phosphorylation of hexoses by adenyl pyrophosphate. Jour. Biol. Chem. 148: 117–126. 1943.
2. Kotelnikova, A. V. The phosphomutase of adenosinediphosphoric acid in animal tissues. Biokhimiya 14: 145–154. 1949.
3. Barkulis, S. S. and Lehninger, A. L. Myokinase and the adenine nucleotide specificity in oxidative phosphorylation. Jour. Biol. Chem. 190: 339–344. 1951.
4. Novikoff, A. B., Hecht, L., Podber, E., and Ryan, J. Phosphatases of rat liver. I. The dephosphorylation of adenosinetriphosphate. Jour. Biol. Chem. 194: 153–170. 1952.
5. Kielley, W. W. and Kielley, R. K. Myokinase and adenosinetriphosphatase in oxidative phosphorylation. Jour. Biol. Chem. 191: 485–500. 1951.
6. Kornberg, A. and Pricer, W. E., Jr. Enzymatic phosphorylation of adenosine and 2,6-diaminopurine riboside. Jour. Biol. Chem. 193: 481–495. 1951.
7. Loomis, W. D. Glutamyl transferase in higher plants. Doctoral dissertation, Univ. of California, Berkeley, California. 1953.
8. Stumpf, P. K. Fat metabolism in higher plants. III. Enzymic oxidation of glycerol. Plant Physiol. 30: 55–58. 1955.
9. Mazelis, M. and Stumpf, P. K. Fat metabolism in higher plants. VI. Incorporation of P^{32} into peanut mitochondrial phospholipids. Plant Physiol. 30: 237–243. 1955.
10. Arnon, D. I. and Whatley, F. A. Factors influencing oxygen production by illuminated chloroplast fragments. Arch. Biochem. 23: 141–156. 1949.
11. Humphreys, T. E. and Conn, E. E. The oxidation of reduced diphosphopyridine nucleotide by lupine mitochondria. Arch. Biochem. Biophys. 60: 226–243. 1956.
12. Cohn, W. E. and Carter, C. E. The separation of adenosine polyphosphates by ion exchange and paper chromatography. Jour. Amer. Chem. Soc. 72: 4273–4275. 1950.
13. Kornberg, A. Reversible enzymatic synthesis of diphosphopyridine nucleotide and inorganic pyrophosphate. Jour. Biol. Chem. 182: 779–793. 1950.
14. Kornberg, A. Enzymatic synthesis of triphosphopyridine nucleotide. Jour. Biol. Chem. 182: 805–813. 1950.
15. Siekevitz, P. and Potter, V. R. Adenylate kinase of rat liver mitochondria. Jour. Biol. Chem. 200: 187–196. 1953.
16. Leuthardt, F. and Bruttin, A. Concerning the myokinase of liver. Helv. Chim. Acta 35: 464–467. 1952.
17. Kielley, W. W. and Kielley, R. K. A specific adenosine triphosphatase of liver mitochondria. Jour. Biol. Chem. 200: 213–222. 1953.
18. Hill, R. and Scarisbrick, R. Production of oxygen by illuminated chloroplasts. Nature 146: 61–62. 1940.
19. Li, L. P. and Bonner, J. Experiments on the localization and nature of tea oxidase. Biochem. Jour. 41: 105–110. 1947.
20. Shaw, M. Phosphorylase in the chloroplasts of wheat. Canadian Jour. Bot. 32: 523–526. 1954.
21. Neish, A. C. Studies on chloroplasts. II. Their chemical composition and the distribution of certain metabolites between the chloroplasts and the remainder of the leaf. Biochem. Jour. 33: 300–308. 1939.
22. Arnon, D. I., Whatley, F. R., and Allen, M. B. Photosynthesis by isolated chloroplasts. II. Photosynthetic phosphorylation, the conversion of light into phosphate bond energy. Jour. Amer. Chem. Soc. 76: 6324–6319. 1954.
23. Strominger, J. L., Heppel, L. A., and Maxwell, E. S. A new mechanism for dephosphorylation of nucleoside di- and triphosphates. I. Transphosphorylation between nucleoside monophosphates and nucleoside triphosphates. Arch. Biochem. Biophys. 52: 488–491. 1954.
24. Berg, P. and Joklik, W. K. Enzymatic phosphorylation of nucleoside diphosphates. Jour. Biol. Chem. 210: 657–672. 1954.
25. Utter, M. F., Kurohashi, K., and Rose, I. A. Some properties of oxalacetic carboxylase. Jour. Biol. Chem. 207: 803–819. 1954.
26. Cardini, C. E., Leloir, L. F., and Chiriboga, J. The biosynthesis of sucrose. Jour. Biol. Chem. 214: 149–155. 1955.
27. Leloir, L. F. and Cardini, C. E. The biosynthesis of sucrose phosphate. Jour. Biol. Chem. 214: 157–165. 1955.
28. Strominger, J. L., Kalckar, H. M., Axelrod, J., and Maxwell, E. S. Enzymatic oxidation of uridine diphosphate glucose to uridine diphosphate glucuronic acid. Jour. Amer. Chem. Soc. 76: 6411–6412. 1954.
29. Kennedy, E. P. and Weiss, S. B. Cytidine diphosphate choline: a new intermediate in lecithin biosynthesis. Jour. Amer. Chem. Soc. 77: 250–251. 1955.
30. Sanadi, D. R., Gibson, D. M., and Ayengar, P. Guanosine triphosphate, the primary product of phosphorylation coupled to the breakdown of succinyl coenzyme A. Biochim. Biophys. Acta 14: 434–436. 1954.
31. Stumpf, P. K. Phosphate assimilation in higher plants. In: Symposium on Phosphorus Metabolism, W. D. McElroy and B. Glass, vol. II. P. 51. Johns Hopkins Press, Baltimore. 1951.

AUXIN-INDUCED WATER UPTAKE BY AVENA COLEOPTILE SECTIONS[1,2]

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It is known that auxin induces the uptake of water by plant tissues. Three principal suggestions have been made concerning the mechanism of such auxin-induced net water uptake. The first proposes that auxin in some manner plasticizes the cell wall. The second suggests that auxin brings about active synthesis of cell wall material. These two mechanisms suppose osmotic entry of water into the cell in response to lowered wall pressure. The third is that auxin brings about a non-osmotic transport of water into the tissue. Thimann (22) has grouped these into two hypotheses in the form of models. One model visualizes a pump (active transport) and the other a piston arrangement (cell wall pressure reduction). Such active transport has been rigorously defined by Rosenberg (21) as the movement of a substance against a chemical potential or concentration gradient, i.e., an uptake of water against a diffusion gradient as defined by Levitt (16). Active transport of water would consist then of water movement against an osmotic gradient. A fourth possibility in principle would be that an auxin-induced increase in the concentration of osmotically active solutes might occur within the cell.

The cells of plant tissues are surrounded by relatively rigid cell walls and one must discuss water movement in terms of diffusion pressure gradients rather than in terms of osmotic pressure. In the terminology proposed by Meyer and Anderson (19) and by Crafts et al (10), DPD = OP − WP where DPD = diffusion pressure deficit, OP = osmotic pressure of cell contents, and WP = wall pressure. The DPD gradient from an external solution to the inside of the tissue can then be expressed as Δ DPD = $DPD_i - DPD_e$ ($DPD_e = OP_e$) where i = internal and e = external. If Δ DPD is negative, the gradient is outward; if Δ DPD is positive, the gradient is inward. To demonstrate a net active water uptake it is necessary to show that water uptake occurs while the purely osmotic Δ DPD is negative. When the external solution is hypertonic, wall pressure becomes zero and $DPD_i = OP_i$. The net uptake of water from hypertonic solution would then involve movement against an osmotic gradient. It will be shown below that cells of Avena coleoptile sections are essentially in diffusion pressure equilibrium with the external solution over a wide range of external solute concentrations both in the presence and in the absence of auxin. There is no detectable net uptake of water from hypertonic solution by this tissue. It is concluded that water uptake by Avena coleoptile sections both in the presence and absence of auxin is a purely osmotic phenomenon.

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[3] Postdoctoral Fellow, National Science Foundation.

MATERIALS AND METHODS

The material used in this work consisted of 5-mm sections cut 2 mm below the apex of Avena (variety Siegeshafer) coleoptiles. The Avena seedlings were grown in vermiculite contained in stainless steel trays and were watered with distilled water. They were harvested when 96 hours old. Only those coleoptiles 30 ± 2.5 mm in length were used. Each part of each experiment was done using 20 sections floated on 20 ml of solution.

Changes in water content of the sections were measured as changes in section length. Net water uptake by coleoptile sections is attended by only small changes in section diameter. Kelly (12) has shown that under conditions similar to those used in elongation studies water uptake in the presence of auxin is linear with time. Measurements of section length were made under a dissecting microscope with a decimillimeter stage micrometer. The variability in elongation rates of sections under the present conditions has been discussed by McRae and Bonner (18).

Three general basal media were used: 1) potassium maleate buffer (0.0025 M, pH 4.8) with or without potassium indoleacetate (5 mg/l); 2) potassium maleate buffer, sucrose (0.09 M), $MnSO_4$ (100 mg/l), arginine (100 mg/l) and with or without potassium indoleacetate (5 mg/l); 3) redistilled water with or without indoleacetic acid (IAA) (5 mg/l, pH 5.0 to 5.5), potassium-free. All solutions were made up with redistilled water.

In the following report, the terms hypotonic and hypertonic are used in referring to solutions external to the cell. These terms are defined as follows: hypertonic = solution whose OP is greater than OP_i; hypotonic = solution whose OP is less than OP_i. It will be shown below that OP_i for freshly cut Avena coleoptile sections under the present conditions is equivalent to approximately 0.4 M mannitol.

EXPERIMENTAL RESULTS

Data on the time course of elongation of sections in media of two different osmotic concentrations are given in figure 1. In these experiments, medium 1 containing buffer alone was used. To this IAA was added as indicated. Mannitol was used as the osmotically active solute. The sections were allowed one hour to come to osmotic equilibrium with the solution before addition of auxin. It is evident that sections do not elongate in response to auxin in an external osmotic concentration of 0.5 M. The use of elevated temperatures and of forced aeration does not increase elongation under conditions of high mannitol concentration.

The respiratory rate of Avena coleoptile sections is depressed in the presence of increasing concentration of an external solute, as is shown in figure 2.

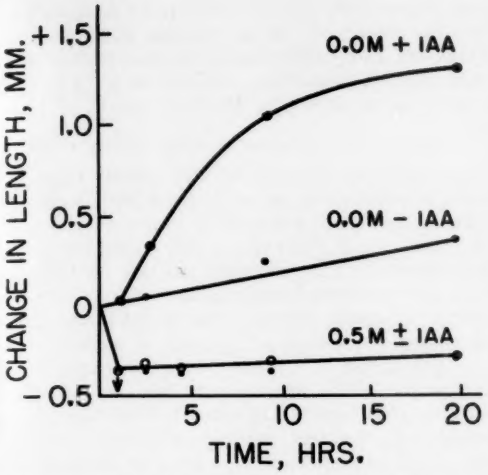

FIG. 1. Effect of external osmotic concentration on elongation of Avena coleoptile sections as a function of time. 0.0025 M potassium maleate, pH 4.8 with or without 0.5 M mannitol, and with or without 5 mg/l IAA. IAA added at arrow.

The rates of oxygen uptake were measured over a period of 3 hours. The presence of IAA increases rate of respiration at low external osmotic concentrations, as has been earlier reported by Commoner and Thimann (9), Bonner (5) and others. The auxin-induced increment in respiration diminishes with increasing external osmotic concentration, and disappears at an OP_e of 0.4 M in which elongation is reduced to a very low value. These results parallel those obtained with Jerusalem artichoke storage tissue in which the respiratory increment induced by IAA disappears as OP_e is increased (6).

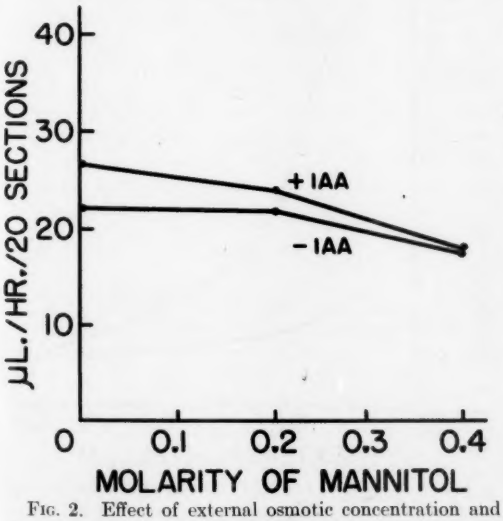

FIG. 2. Effect of external osmotic concentration and of IAA on oxygen uptake of Avena coleoptile sections. 0.0025 M potassium maleate buffer, pH 4.8, 5 mg/l IAA.

It is known that the response of coleoptile sections to IAA is prolonged in time by the addition of sucrose or certain other substances to the media. Figure 3 gives data on the time course of the elongation response to IAA in the more complete basal medium containing sucrose, manganese and arginine, and containing added mannitol to produce varying osmotic pressures. The sections were first equilibrated in the medium for one hour, after which auxin was added. In the basal medium alone (OP_e = 0.09 M) elongation starts at once when IAA is added. At

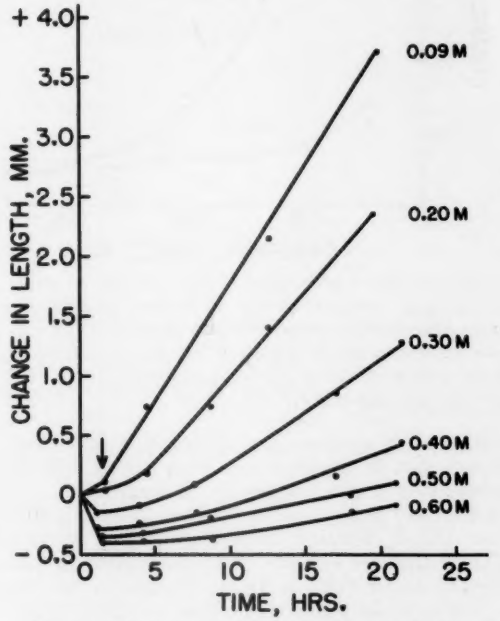

FIG. 3. Effect of external osmotic concentration on elongation of Avena coleoptile sections as a function of time. 0.09 M sucrose plus various concentrations of mannitol, 100 mg/l arginine, 100 mg/l $MnSO_4$, 0.0025 M potassium maleate, pH 4.8. IAA (5 mg/l) added at arrow.

higher values of OP_e, a lag period in attainment of steady state elongation is evident. The length of this lag period is greater the larger the value of OP_e. The final steady state elongation rates also decrease regularly as OP_e is increased. Figure 4 summarizes data on the elongation of sections in the presence or absence of IAA and in media of varying osmotic concentrations. The effects of increasing external osmotic concentration on endogenous elongation (elongation in the absence of added IAA) do not parallel effects on the auxin-induced elongation of these sections. The endogenous elongation exhibits the initial non-linear decline with increasing OP_e at a lower concentration than does elongation in response to added IAA. The two coincide at an external osmotic con-

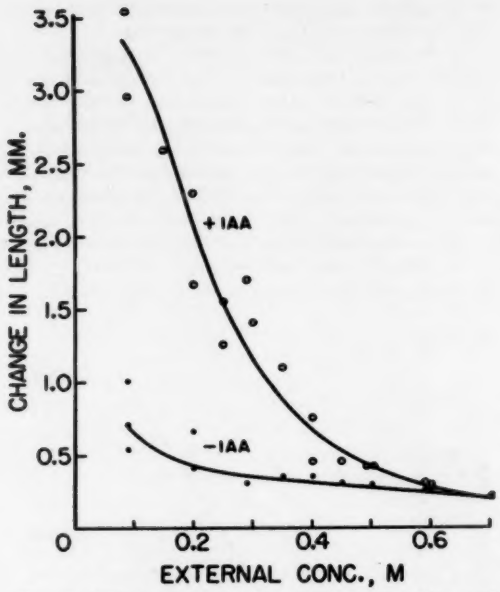

FIG. 4. Effect of external osmotic concentration and of auxin on total elongation of Avena coleoptile sections. Incubation time 20 hrs. 0.09 M sucrose plus various concentrations of mannitol, 100 mg/l arginine, 100 mg/l $MnSO_4$, 0.0025 M potassium maleate, pH 4.8, with or without 5 mg/l IAA.

centration of approximately 0.7 M. The cells of sections placed in solutions more concentrated than approximately 0.4 M are rapidly plasmolyzed, as will be discussed in detail below. After a 20-hour incubation period, however, deplasmolysis has occurred. This is true, for example, of sections placed in solutions as concentrated as 0.6 M. Elongation has then occurred in the presence of solutions initially hypertonic to the 0.4 M osmotic concentration of the initial sections.

Burström (7) has shown that the irreversible extension of wheat roots is independent of OP_e in the hypotonic region. This would suggest that the cell elongation of such roots is not due to a mere passive stretching of the wall and favors some hypothesis relating active wall synthesis to cell enlargement. In order to determine if a similar situation obtains for the Avena coleoptile, sections were placed in plasmolyzing solutions for one hour after 20 hours of elongation. The difference between initial plasmolyzed length (plasmolyzed at zero hours) and final plasmolyzed length (plasmolyzed at 20 hours) is taken as the irreversible elongation in accordance with Burström's definition. The data for these experiments are given in figure 5. The irreversible component, like the total elongation of Avena coleoptile sections, is an inverse function of OP_e. The Avena coleoptile is not, therefore, similar in behavior to Burström's wheat roots as far as irreversible extension is concerned. In the case of the Avena coleoptile, the greater the turgor pressure the greater the elongation rate. This implies that something more than intimate contact of cytoplasm with the cell wall is essential to increase in coleoptile length.

Osmotic Quantities during Elongation

In order to ascertain to what extent purely osmotic considerations govern auxin-induced water uptake in the Avena coleoptile it is necessary to have measurements of DPD and of OP_i during the elongation process. The simplified method of Ursprung (25) was used for determination of the DPD_i of Avena coleoptile tissue. The method consists in measuring the lengths of a group of sections which are then placed in a graded series of mannitol concentrations. The sections are held submerged in stainless steel baskets and argon bubbled through the solutions to maintain anaerobic conditions. A separate group of sections is used for each mannitol concentration. After one to two hours, the sections are removed and their lengths quickly measured. That group which shows no change in length is said to have been in a solution whose $OP = DPD_i$. The maintenance of anaerobic conditions during the DPD determination insures to a high degree that metabolic processes such as are essential to IAA induced net water uptake are kept at a minimum and that any such metabolic components of DPD are not measured.

According to classical osmotic theory, the simplified method of Ursprung should also be capable of yielding values of OP_i for the tissue measured. The cytoplasm should pull away from the cell wall at incipient plasmolysis and as OP_e is further increased, the cytoplasm should continue to contract without

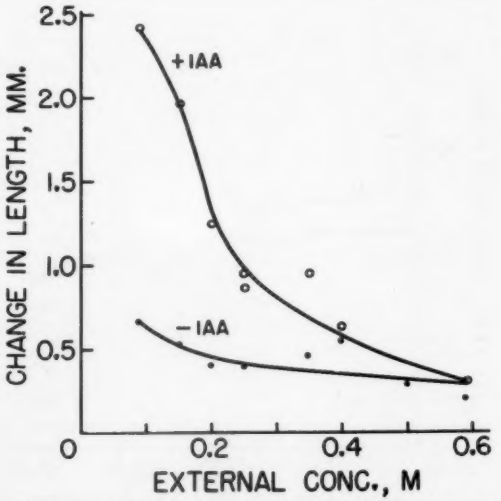

FIG. 5. Effect of external osmotic concentration and auxin on irreversible elongation of Avena coeloptile sections. Incubation time 20 hrs followed by plasmolysis in 1 M mannitol. Same experimental conditions as figure 4.

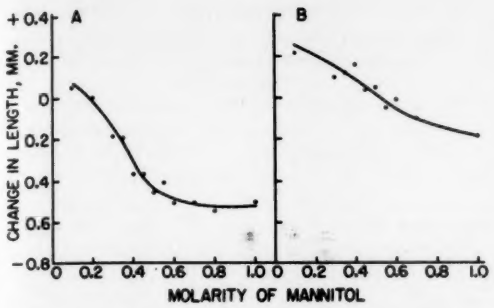

FIG. 6 A. Change in length of Avena coleoptile sections as a function of external mannitol concentration. Incubation for 1 to 2 hours under anaerobic conditions. Freshly cut sections.

FIG. 6 B. Change in length of Avena coleoptile sections as a function of external mannitol concentration. Incubation for 1 to 2 hrs under anaerobic conditions. Sections previously grown 20 hrs in complete medium of osmotic concentration 0.5 M (mannitol + sucrose) solution and containing 5 mg/l IAA.

further cell shrinkage. Hence, the curve which relates tissue length (or change in length) to OP_e should show a sharp inflection at the OP_e of incipient plasmolysis, and should become a straight line of zero slope for higher values of OP_e. Ketellapper (13) has previously attempted to determine the osmotic concentration of Avena section tissue in this way. His data appear to follow closely the expectations of classical osmotic lore. This is not true in the present experiments. Figure 6 A presents data on the length of freshly excised Avena sections after one hour of anaerobic incubation in media of varying OP_e. The length of the sections decreases with increasing OP_e up to an external concentration of approximately 0.6 M. Although there is an inflection in the curve between 0.4 and 0.6 M, there is no sharp change in slope. OP_i apparently lies between 0.4 and 0.6 M but cannot be determined more precisely by this method. The same is true for tissue which has been previously incubated in a medium of high OP_e. Figure 6 B gives data on tissue incubated anaerobically for one hour in media of varying OP_e after 20 hours in solution containing IAA in 0.5 M solution (mannitol plus sucrose). There is no evident inflection in the curve of figure 6 B. It is necessary to conclude, therefore, that OP_i cannot be measured in this way for Avena coleoptile section tissue. This method is, however, satisfactory for DPD determinations, and yields a precision of determination of approximately ± 0.05 M. Table I gives DPD values for sections after varying pretreatment. The data of table I show that DPD_i as measured by the short term anaerobic incubation method is always equal to the OP_e of the external solution in which the section has previously been incubated. Equilibration of tissue DPD with external OP appears to take place within one hour over a wide range of OP_e values. Equilibration of DPD_i and OP_e is maintained through a 20-hour period both in the presence and absence of added auxin. There is no evidence in these data for any component of DPD_i dependent on aerobic metabolism and measurable by the present method. This important point was further studied by experiments in which argon was bubbled through solutions in which sections had previously been elongating under the influence of auxin. The change of section length with time in argon for

TABLE I

DIFFUSION PRESSURE DEFICIT (DPD) OF AVENA COLEOPTILE SECTIONS AFTER INCUBATION FOR 1 HR AND 20 HRS IN MEDIA OF DIFFERENT OSMOTIC CONCENTRATIONS

EXTERNAL CONC, M	DPD OF SECTION TISSUE, M		
	AFTER 1 HR	AFTER 20 HRS	
		+IAA	−IAA
0.25	0.25	0.27	0.28
0.35	0.36	0.39	0.40
0.40	0.37	0.41	0.46
0.50	0.47	0.52	0.45
0.59	0.60	0.54	0.56

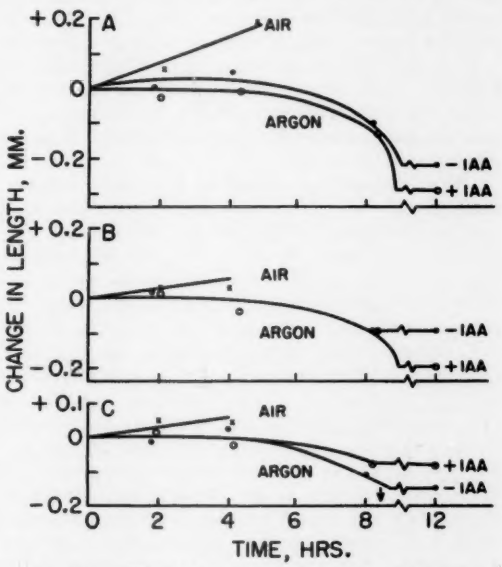

FIG. 7 A. Effect of anaerobic conditions (argon) on change in length of Avena coleoptile sections in 0.4 M solution (0.0025 M potassium maleate, pH 4.8, 100 mg/l arginine, 100 mg/l $MnSO_4$, 0.09 M sucrose, mannitol, with or without 5 mg/l IAA) following 20 hrs in same solution under aerobic conditions. Sections transferred to distilled water at arrow indicated in figure 7 C.

FIG. 7 B. Effect of anaerobic conditions on change in length of sections in 0.5 M solution. Conditions same as in figure 7 A except for mannitol concentration.

FIG. 7 C. Effect of anaerobic conditions on change in length of sections in 0.6 M solution. Conditions same as in figure 7 A except for mannitol concentrations.

sections in solutions of several different values of OP_e are plotted in figure 7 A, B and C. No change in section length occurs during the first four hours after conditions are made anaerobic. After 4 hours the sections begin to shrink. This is because the tissue is killed by the prolonged anaerobic incubation as shown by microscopic examination and lack of section expansion after transfer to distilled water. Two hours of anaerobiosis are, however, evidently without deleterious effects. These data tend to confirm the conclusion that the tissue is in DPD equilibrium with the external solution at all times, and that IAA-induced elongation occurs in the absence of any apparent DPD gradient over the entire range of external osmotic concentrations used. Since water enters the cell under the influence of auxin it must do so under the influence of a positive DPD gradient. This is apparently small and not detectable by the present methods of measurement.

When sections are placed in hypertonic solution, their cells are rapidly plasmolyzed. The cells of such sections subsequently deplasmolyze under aerobic conditions. One might, therefore, conclude that absorption of solutes or production of solutes within the tissue has taken place. According to this view, the sections deplasmolyze because OP_i is increased and the solution is no longer hypertonic. At the same time, wall pressure increases above zero so that DPD remains constant while OP_i increases. Le Gallais (14) has shown that the OP of sap expressed from Avena coleoptile section tissue increases proportionally to external osmotic concentration regardless of solute used and both in the presence and absence of auxin. Le Gallais' work was done with the cryoscopic method of osmotic pressure determination. His results suggest, however, that the cryoscopic values for OP_i are erroneously high because the sap expressed includes plasmolytic solution which has entered the tissue without entering the vacuole. The cryoscopic technique is not, therefore, useful for the present purpose. That an increase in OP_i does take place during incubation of sections in initially hypertonic solutions can however be shown by the plasmolytic method of OP determination. This method consists in the examination of coleoptile sections from the DPD determination solutions under the high power of the microscope. Cells are counted through the median region of the layer of cells just below the outer epidermis. OP_i is taken as equal to the OP_e of that solution in which sections have 50 % of their cells plasmolyzed.

The initial OP_i of freshly cut Avena coleoptile cells was found to be 0.42 M. Sections incubated for 20 hours yield various values of OP_i, depending on the solution used for incubation. Results of typical experiments are summarized in table II. In the first place, sections incubated in water alone (no solute) decrease in OP_i if IAA is present. This effect is apparently due to dilution of cell contents by the water taken up under the influence of auxin as has been previously described. Sections in mannitol alone show small increases in OP_i over the 20-hour period. Since some elongation occurs in mannitol, a small amount of mannitol must have entered the tissue. The addition of sucrose to the medium, however, permits very large increases in OP_i to take place. This is true both for solutions which are initially hypotonic and for those which are initially hypertonic (with added mannitol). The osmotic pressure increase in mannitol plus sucrose is larger than that in sucrose alone in the presence of IAA. This is undoubtedly due to the greater dilution of the cell contents in the latter case as pointed out by Hackett (11) for potato tissue. The presence of sucrose in the medium evidently permits the tissue to make an extensive adjustment of OP_i.

TABLE II

INTERNAL OSMOTIC PRESSURE OF AVENA COLEOPTILE SECTIONS AFTER INCUBATION IN VARIOUS SOLUTIONS

TREATMENT	INTERNAL OSMOTIC PRESSURE (OP_i), M	
	+IAA	-IAA
Redistilled H_2O	0.32	0.42
0.0813 M Sucrose	0.53	0.68
0.59 M Mannitol	0.44	0.45
0.5 M Mannitol + 0.0813 M sucrose	0.69	0.69
0.09 M Mannitol	0.43	0.47
0.09 M Ethylene glycol	0.42	0.52
0.045 M NaCl	0.45	0.60

Incubation: 10 hrs for H_2O treatment, 20 hrs for other treatments. Initial OP_i = 0.42 M.

The data of figure 8 further concern the question of the role of sucrose in the elongation of Avena coleoptile sections. Sections were incubated for 29 hours in initially hypertonic solutions made up of mannitol or sucrose and mannitol, and with or without IAA. The sections were initially plasmolyzed by the hypertonic solutions in all cases. Deplasmolysis only occurred with sections incubated in the presence of sucrose. Sucrose must therefore contribute to the

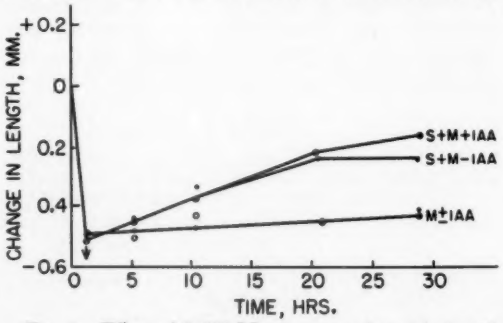

FIG. 8. Effect of 0.0813 M sucrose on change in length of Avena coleoptile sections in initially hypertonic solution containing mannitol in concentrations such that total molarity is 0.59 M. IAA added at arrow. 5 mg/l K-free IAA, no buffer. Final pH 5.5.

increase in OP_i of the tissue. Sections which have deplasmolyzed elongate more slowly than those which have never been plasmolyzed in agreement with the similar findings of Ketellapper (13). Plasmolysis itself apparently causes some irreversible damage.

It has been shown above that the presence of a small amount of sucrose in the medium permits sections to deplasmolyze in an otherwise hypertonic concentration of mannitol. The presence of sucrose similarly maintains OP_i and rate of water uptake in the presence of auxin. The following type of experiment was done to find out whether sucrose pretreatment can maintain subsequent elongation rate in hypotonic mannitol solution. Sections were pretreated in 0.09 M sucrose with IAA for 4 hours and were then transferred to 0.09 M mannitol and IAA for periods up to 20 hours. The results are given in figure 9.

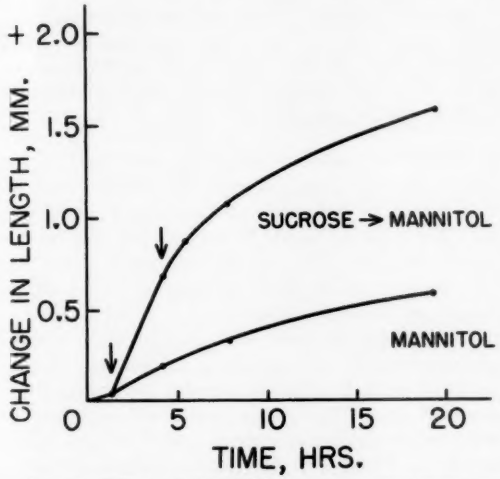

FIG. 9. Effect of 0.0813 M sucrose pretreatment on subsequent elongation of Avena coleoptile sections in 0.09 M mannitol. IAA added at first arrow. Transfer to mannitol at second arrow. 5 mg/l K-free IAA, no buffer; final pH 5.5.

The elongation rate with the latter solution drops off rapidly with time. The initial elongation rate in sucrose is greater than that in an equi-osmolar concentration of mannitol. The initial rate following transfer from sucrose to mannitol is greater than the initial rate for sections maintained in mannitol alone, but the effect does not persist. Apparently the continuing presence of sucrose is required for maintenance of elongation rate. This is consistent with the idea first presented by Thimann and Schneider (24) that sucrose acts in part to maintain OP_i at a constant high level during auxin-induced elongation.

It is of interest to know what other solutes can replace sucrose in its role of maintaining elongation rate and, apparently, OP_i. A variety of permeating substances were used, each in a concentration of 0.01 M. The results, presented as progress curves of elongation, are given in figure 10. Glucose is nearly

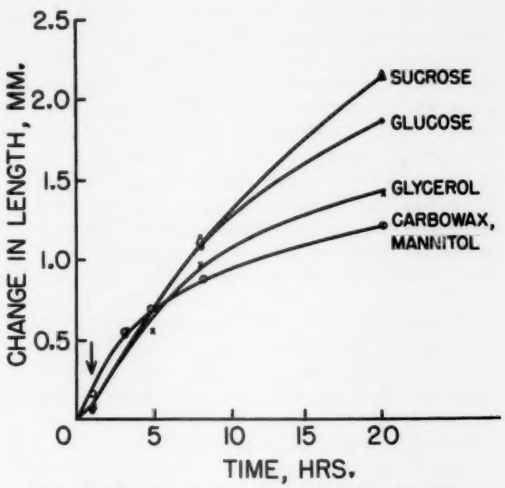

FIG. 10. Change in length of Avena coleoptile sections as a function of time and in the presence of several different solutes. Each solute given at 0.01 M concentration. IAA added at arrow. No buffer, 5 mg/l K-free IAA, final pH 5.5.

as effective as sucrose. Glycerol is but little superior to mannitol in the support of continued elongation rate. Table III gives further data on the elongation rates of sections in solutions containing various solutes, all tested at a higher concentration, 0.09 M (0.045 M for KCl and NaCl). At this concentration, sucrose and glucose are considerably more effective than any of the other solutes tested. It may be seen that the substances used form a series of varying effectiveness in replacing sucrose or glucose. Even an apparently non-metabolizable substance such as ethylene glycol is one third as effective as sucrose. NaCl and KCl possess an intermediate status.

TABLE III

EFFECT OF VARIOUS SOLUTES ON IAA-INDUCED ELONGATION OF AVENA COLEOPTILE SECTIONS

SOLUTE	ELONGATION, MM
Sucrose (0.0813 M)	2.86
D-Glucose	2.79
NaCl (0.045 M)	2.23
KCl (0.045 M)	1.75
D-Raffinose	1.16
Glycerol	1.01
Ethylene glycol	0.90
D-Ribose	0.87
Urea	0.77
L-Rhamnose	0.70
D-Xylose	0.61
L-Sorbose	0.55
L-Arabinose	0.23
D-Arabinose	0.22

Solutions 0.09 M except where noted. Incubation 20 hrs. 5 mg/l IAA throughout. Media unbuffered pH 5.4 to 6.1 for all solutions except that containing urea in which pH was 7.0. Elongation above mannitol control.

The absolute elongation of sections in 0.09 M solution is depressed as compared to that in 0.01 M solution for all solutes tested except sucrose and glucose. These two substances are more effective in increasing elongation rate in the 0.09 M concentration than at 0.01 M. This is undoubtedly due in part to the fact that sucrose and glucose are metabolizable as shown by Bonner (4). In addition, however, sections incubated in IAA sucrose-containing solutions show an increase in OP_i as compared to similar sections incubated in mannitol or in glycol, a readily absorbed solute (table II). In the absence of IAA, OP_i is increased somewhat above initial values. The relatively large increases in OP_i in the presence of sucrose may be due to the formation by the tissue of smaller molecules from the large sucrose molecules taken in. In any case, the osmotic readjustment caused by sucrose appears to be independent of the presence or absence of IAA (table II).

It has been noted above that when Avena coleoptile sections are placed in solutions of high external osmotic concentration there is a lag period in the attainment of steady state auxin-induced elongation. The experiments outlined above have suggested that this lag period may represent the time needed by the cells of the tissue to accumulate solutes and to increase in OP_i. The following experiments were carried out to further characterize the effects of different external solutes on the lag period. Sections were placed in hypotonic solution, 0.2 M, containing mannitol alone or mannitol with a small amount of sucrose or NaCl. All solutions contained IAA. The sections were left in these solutions for four hours to permit any osmotic readjustment to occur. The sections were then transferred to an equi-osmolar solution of mannitol, sucrose and IAA. The data of figure 11 show that sections pretreated for four hours in mannitol alone show a lag period in attainment of steady state elongation when transferred to mannitol, sucrose and IAA. If the pretreatment included sucrose or KCl in addition to mannitol the sections start elongating immediately and rapidly attain the final steady state rate. Apparently, the lag period does represent a period of readjustment of internal osmotic concentration. The length of the lag period is dependent on the presence in the external solution of readily absorbable solutes. It may be noted that NaCl behaves as does KCl in permitting shortening of the lag period.

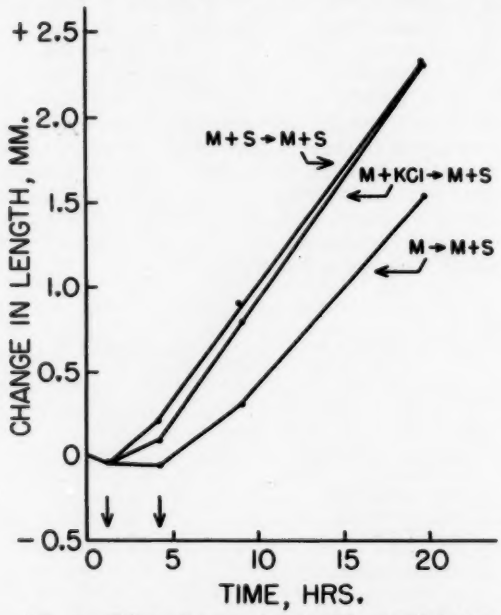

FIG. 11. Effect of pretreatment with mannitol alone or mannitol in the presence of KCl or sucrose on subsequent elongation of Avena coleoptile sections in mannitol and sucrose. Total solute concentration 0.2 M. IAA added at first arrow. Transfer to mannitol and sucrose at second arrow. 5 mg/l K-free IAA, no buffer, final pH 5.0–5.2.

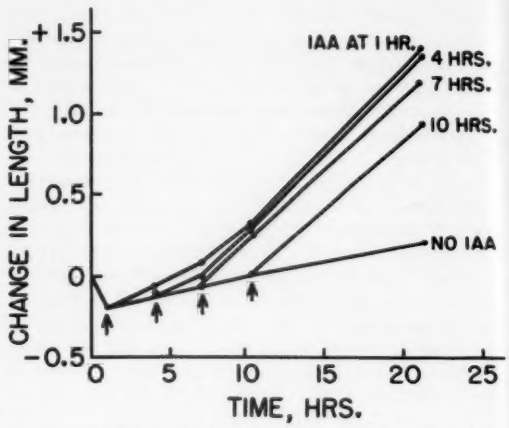

FIG. 12. Effect of time of addition of IAA on subsequent elongation of Avena coleoptile sections in solution of 0.3 M total concentration. IAA added at arrows. 0.0813 M sucrose, 0.21 M mannitol, 0.0025 M potassium maleate, pH 4.8, 5 mg/l IAA.

The osmotic regulation which takes place during the lag period in solutions of moderate osmotic concentration is not dependent upon the presence of auxin in the external medium. In order to test the effect of auxin on the lag process, sections were incubated in 0.3 M solutions containing mannitol and sucrose. Auxin was added at various times and the elongation rate after addition of auxin was followed. The data are presented in figure 12. It is apparent that the lag period extends for about 7 hours and that its duration is independent of the presence or absence of auxin in the external solution.

Discussion

An important conclusion of the present work is that the auxin-induced uptake of water by Avena coleoptile sections follows osmotic principles. It is of interest to consider these results in relation to earlier reports of metabolically maintained DPD gradients.

In order to determine whether a tissue is able to accumulate water against a diffusion pressure gradient it is necessary to possess information concerning the osmotic pressure (OP_i) of the tissue. The simplified method of Ursprung (25) has been shown above to be inapplicable because of the absence of a clear-cut inflection in the volume-OP_e relation as OP_e is increased. Coleoptile tissue as well as other tissues discussed by Crafts et al (10) continue to shrink as OP_e is increased above OP_i. The cryoscopic technique when applied to tissue sections which have been in contact with plasmolyzing solutions is attended by serious errors in assessment of OP_i as has been shown by Le Gallais (14). In the case of plasmolyzed tissues, for example, the cryoscopic method yields values of apparent OP_i which are too high. The plasmolytic method, however, can be used for OP_i determinations even with tissues which have been incubated in initially hypertonic solutions.

Bogen (2) has shown that leaf tissues deplasmolyze under aerobic conditions in sucrose solutions in which they are initially plasmolyzed. A similar result is reported here. In the present case it has been shown that sucrose penetrates the Avena coleoptile sufficiently rapidly to permit of deplasmolysis in less than 20 hours and that the deplasmolysis is an osmotic one. Bogen's experiment would appear to be susceptible to a similar interpretation. It has been reported by Bonner et al (6) that Jerusalem artichoke discs can take up water under the influence of auxin from solutions which are initially hypertonic. Although the determinations of initial OP_i are difficult with this tissue they can be made and the values reported earlier have been confirmed. Determinations of OP_i after a period of water uptake are still more difficult to perform with Jerusalem artichoke tissue and are more questionable. Burström, (8) who used cryoscopic techniques, found that OP_i of such tissue increases with time in initially hypertonic solutions. The cryoscopic technique requires an elaborate correction if it is to be used under these conditions. Nonetheless, it is probable that the OP_i of Jerusalem artichoke increases with time in hypertonic solutions as shown in the present paper for Avena. All of the experiments of Bonner et al were done in the presence of an absorbable solute, namely potassium phosphate buffer. The water loss which takes place when Jerusalem artichokes in initially hypertonic solution (0.2 M mannitol) are transferred to anaerobic conditions may be due to progressive tissue damage as it is with Avena coleoptile sections.

The most nearly valid evidence, at least as a first approximation, of water uptake against a diffusion pressure gradient in higher plants is contained in the work of van Overbeek (20). It was found by van Overbeek that the exudate of an excised tomato root-shoot system may have an osmotic concentration 1 atmosphere less than that of the solution bathing the root. Arisz et al (1) using more refined techniques of measurement reduced this difference to 0.5 atmosphere or less. The latter workers propose that the lower OP of the exudate is due to salt absorption from the sap between the root and the stump as well as to dilution by water from tissues adjacent to the xylem. Furthermore, if the zone of water absorption exceeds the zone of salt absorption, dilution of the sap would occur.

Neither the present work, nor other work reported on water, clearly support the concept of active uptake as defined by Rosenberg (21) and Levitt (16). It is clear however that the performance of auxin-induced water uptake is attended by an increase in respiratory rate and that the auxin-induced increment is abolished by external solute concentrations which abolish net water uptake. The increase in respiration caused by auxin does not then have to do with water accumulation against a water concentration gradient but would appear rather to be related in some way to the primary act involved in cell elongation.

It has long been known that sugars (3) or potassium salts (24) act as co-factors in auxin-induced elongation of coleoptile sections. The role of these substances is in part an osmotic one. They contribute to the maintenance of OP_i. The presence of sucrose in the medium extends the period over which elongation is linear with time. This effect cannot be achieved by sucrose pretreatment as shown by experiments in which sections decrease in elongation rate after transfer to sucrose-free solutions. The effect of sucrose on rate of water uptake may be due in part to the utilization of the material in respiration and in the support of cellular syntheses. In addition however sucrose clearly provides internal osmotically active material both as intact sucrose molecules and perhaps as metabolically produced smaller molecules. The lesser effectiveness of salts and especially of other sugars is presumably due to their being less effective as food material and less abundantly converted to smaller molecules.

It has been concluded that in the Avena coleoptile DPD_i is at all times essentially in equilibrium with OP_e and that by the methods presently available no detectable differences between these two quantities can be found. Within the limits of measurement of DPD ($\pm$ 0.05 M) no movement of water against a DPD gradient appears to take place with Avena coleoptile sections. When non-absorbable solute is used to constitute a hypertonic solution, no elongation of the tissue takes place. It is therefore highly unlikely that active water uptake is a factor in auxin-induced elongation in this tissue. This agrees with the conclusions reached earlier with other tissues by Levitt (15, 17) and by Thimann (23). Adjustments

of osmotic concentration take place in the coleoptile section provided that an absorbable solute is present in the medium. These osmotic adjustments are however independent of auxin and occur in the absence as well as in the presence of added growth substance.

Since auxin-induced water uptake is a purely osmotic phenomenon in the Avena coleoptile and since auxin does not appear to directly influence internal osmotic concentration, it may be concluded that auxin must in some way decrease cell wall pressure. The fact that the irreversible component of the total elongation behaves as an inverse function of OP_e in the hypotonic region is consistent with the hypothesis that cell wall plasticization rather than cell wall synthesis is a primary cell wall effect. This is in contrast to the state of affairs with wheat roots (7) in which irreversible extension is independent of OP_e in the hypotonic region as reported by Burström. In any case it would appear that a detailed investigation of cell wall metabolism as related to the presence or absence of auxin may be required for the further elucidation of the mechanism of auxin action.

Summary

Avena coleoptile sections are in apparent diffusion pressure equilibrium with the external solution at all times, i.e., there is no detectable aerobic metabolic component of DPD in either hypertonic or hypotonic solution.

Both total elongation and irreversible elongation are inverse functions of external osmotic concentration in the hypotonic region.

Tissue placed in initially hypertonic solution deplasmolyzes and grows if a sufficient concentration of an absorbable solute such as sucrose is present in the external solution. It has been shown that the role of the permeating solutes is to cause an increase in internal osmotic concentration of the tissue.

Tissue in hypotonic solution maintains or increases in internal osmotic concentration as elongation occurs, provided that absorbable solute is present in the external solution. Tissue maintained in distilled water exhibits a decrease in internal osmotic concentration as elongation occurs.

When Avena coleoptile sections are placed in slightly hypotonic or in hypertonic solution there is a lag period in the attainment of steady state elongation rate. The duration and intensity of this lag period increases with external osmotic concentration. The length of the lag period is independent of the presence of auxin and is determined by the time required by the section to make an osmotic readjustment to the external solution by absorption of solutes.

Auxin-induced water uptake in Avena coleoptile sections does not appear to be due to the effect of auxin on maintenance of a metabolically controlled component of internal diffusion pressure deficit or to effects on the osmotic concentration of the tissue. The present evidence points to effects of auxin on the cell wall as an important factor in auxin-induced cell elongation.

Literature Cited

1. Arisz, W. H., Helder, R. J., and van Nie, R. Analysis of the exudation process in tomato plants. Jour. Exptl. Bot. 2: 257–297. 1951.
2. Bogen, H. J. Beiträge zur Physiologie der nichtosmotischen Wasseraufnahme. Planta 42: 140–155. 1953.
3. Bonner, J. The action of the plant growth hormone. Jour. Gen. Physiol. 17: 63–76. 1933.
4. Bonner, J. Biochemical mechanisms in the respiration of the Avena coleoptile. Arch. Biochem. 17: 311–326. 1948.
5. Bonner, J. Relations of respiration and growth in the Avena coleoptile. Amer. Jour. Bot. 36: 429–436. 1949.
6. Bonner, J., Bandurski, R. S., and Millerd, A. Linkage of respiration to auxin induced water uptake. Physiol. Plantarum 6: 511–522. 1953.
7. Burström, H. Studies on growth and metabolism of roots. IX. Cell elongation and water absorption. Physiol. Plantarum 6: 260–274. 1953.
8. Burström, H. Growth and water absorption of Helianthus tuber tissue. Physiol. Plantarum 6: 685–691. 1953.
9. Commoner, B. and Thimann, K. V. On the relation between growth and respiration in the Avena coleoptile. Jour. Gen. Physiol. 24: 279–296. 1941.
10. Crafts, A. S., Currier, H. B., and Stocking, C. R. Water in the Physiology of Plants. Pp. 1–240. Chronica Botanica, Waltham, Massachusetts. 1949.
11. Hackett, D. P. The osmotic change during auxin-induced water uptake by potato tissue. Plant Physiol. 27: 279–284. 1952.
12. Kelly, S. The relationship between respiration and water uptake in the oat coleoptile. Amer. Jour. Bot. 34: 521–526. 1947.
13. Ketellapper, H. J. The mechanism of the action of indole-3-acetic acid on the water absorption by Avena coleoptile sections. Acta Bot. Néerl. 2: 388–444. 1953.
14. Le Gallais, D. R. A study of growth of plant tissue in relation to composition and concentration of the external medium. Ph.D. Thesis, University of California, Berkeley. 1955.
15. Levitt, J. The thermodynamics of active (non-osmotic) water absorption. Plant Physiol. 22: 514–525. 1947.
16. Levitt, J. The role of active water absorption in auxin-induced water uptake by aerated potato discs. Plant Physiol. 23: 505–515. 1948.
17. Levitt, J. Further remarks on the thermodynamics of active (non-osmotic) water absorption. Physiol. Plantarum 6: 240–252. 1953.
18. McRae, D. H. and Bonner, J. Chemical structure and antiauxin activity. Physiol. Plantarum 6: 485–510. 1953.
19. Meyer, B. S. and Anderson, D. B. Plant Physiology. Pp. 1–696. D. Van Nostrand Co., New York. 1939.
20. van Overbeek, J. Water uptake by excised root systems of the tomato due to non-osmotic forces. Amer. Jour. Bot. 29: 677–683. 1942.
21. Rosenberg, T. The concept and definition of active transport. Symposia Soc. Exptl. Biol. 8: 27–41. 1954.
22. Thimann, K. V. Studies on the physiology of cell

enlargement. Growth 15 (Suppl.): 5–22. Tenth Symposium, Soc. Study Develop. & Growth, Northampton, Massachusetts. 1951.
23. THIMANN, K. V. The physiology of growth in plant tissues. Amer. Scientist 42: 589–606. 1954.
24. THIMANN, K. V. and SCHNEIDER, C. L. The role of salts, hydrogen-ion concentration and agar in the response of the Avena coleoptile to auxins. Amer. Jour. Bot. 25: 270–280. 1938.
25. URSPRUNG, A. Zur Kenntnis der Saugkraft. VII. Eine neue vereinfachte Methode zur Messung der Saugkraft. Ber. deut. bot. Ges. 41: 338–343. 1923.

PERMEABILITY OF AVENA COLEOPTILE SECTIONS TO WATER MEASURED BY DIFFUSION OF DEUTERIUM HYDROXIDE[1,2]

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The present investigation, carried out in connection with studies of the mechanism by which auxin induces net water uptake, concerns the resistance of Avena coleoptile section tissue to water movement. The factors which govern tissue permeability to water have also been examined. Heavy water has been used in order to eliminate changes in the geometry of the tissue during the measurement. Permeability constants obtained by this method are true diffusion constants in contrast to the filtration constants obtained by osmotic methods. The first use of deuterium oxide (D_2O, 30 to 50 %) solutions in the study of water permeability of plants was that of Wartiovaara (11) on the alga Tollypellopsis. Buffel (2) and Ketellapper (5) have also used 50 % D_2O to determine the water permeability of Avena coleoptiles. These workers studied the outward diffusion of D_2O from tissue previously equilibrated with the material. In both investigations cartesian diver techniques were used for determination of D_2O. The high concentrations of D_2O used exert injurious effects on the tissue after several hours. Buffel therefore allowed his sections to age in distilled water for 12 to 18 hours, a procedure found empirically to decrease such injury.

Buffel (2) has reported that indoleacetic acid (IAA) increases D_2O permeability of sections aged in this manner. Ketellapper (5) on the other hand finds that IAA has slight if any effect on D_2O permeability. It will be shown below that rate of absolute water movement in the coleoptile is rapid and is not influenced by IAA, metabolic inhibitors, or the state of turgor of the tissue.

MATERIALS AND METHODS

The material used in this work consisted of 5-mm sections cut 2 to 3 mm below the apex of the Avena coleoptile. The primary leaf was removed. The Avena seedlings were grown at 25° C in vermiculite contained in stainless steel trays and were watered with distilled water. They were harvested when 96 hours old. Only those coleoptiles 30 ± 2.5 mm in length were used. Each part of each experiment was performed using 50 or 75 sections in 10 or 15 ml of solution respectively.

Dilute solutions of deuterium hydroxide (DHO) (0.5 % D_2O) were prepared from 99.5 % D_2O obtained from Stuart Oxygen Company by authorization of the U. S. Atomic Energy Commission. The D_2O was used without further purification. Groups of sections were placed in DHO solution for varied periods of time. After the desired time interval the group of sections was dried externally with filter paper and placed in a ground glass stoppered vial. The vial was then utilized as the distilling vessel of a micro-distillation apparatus in which the tissue water was removed by lyophilization. The DHO content of the tissue water was determined by mass spectrometry. The direct determination of DHO in the mass spectrometer has been described by Washburn et al (12). The precision of measurement is greater than $\pm$ 0.01 atom percent over the range of DHO concentrations used in this work. The data are presented as mole percent DHO in the tissue water as a function of time.

Indoleacetic acid (IAA), 5 mg/l, was used as the auxin throughout. The basal medium contained potassium maleate buffer (0.0025 M, pH 4.8). All solutions were made up with redistilled water.

RESULTS

The data in figure 1 show that IAA has no influence on the course of entrance of heavy water into coleoptile tissue. The half-time of the movement, i.e., the time required for the tissue to attain a concentration of DHO equal to one half the difference between the external concentration and the initial internal concentration (that of normal water, 0.02 % DHO), is about 9 minutes. This is slightly longer than the half times found by Buffel (2) and Ketellapper (5) of 4 to 7 minutes and 4 to 6 minutes respectively. The logarithmic shape of the curve of figure 1 indicates that water enters the tissue by a simple diffusion process.

The course of the outward diffusion of water was compared with that of the inward diffusion by pretreating tissue for 2 hours in 1.1 % DHO. The tissue

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[3] Postdoctoral Fellow, National Science Foundation.

was then transferred to water (not enriched in deuterium) for varying periods of time. The data of figure 2 show that within experimental error there is no difference in either the shapes of the inward and outward diffusion curves or in the half-times of the diffusion. There is thus no apparent differential between rates of inward and outward diffusion in the presence or absence of IAA.

In the experiments described above water diffuses into the tissue and approaches equilibrium with the external solution. Danielli (3) has presented the concept of facilitated diffusion to cover those cases in which substances move until equilibrium is attained

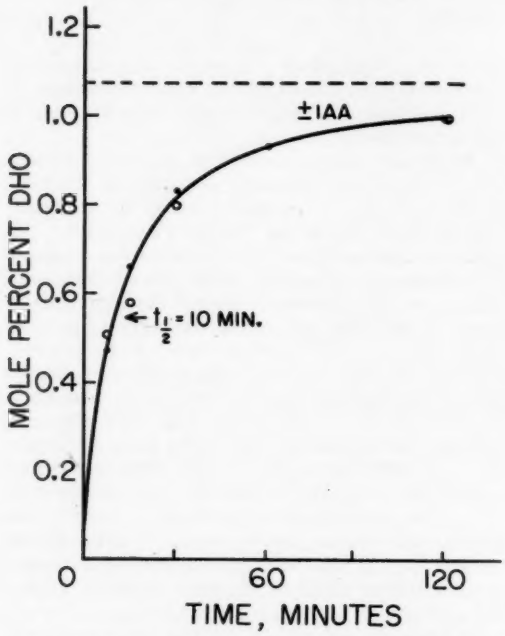

FIG. 1. Effect of 5 mg/l IAA on inward diffusion of DHO into Avena coleoptile sections. External solution concentration indicated by horizontal line. Half-time given at arrow. 0.0025 M K maleate pH 4.8 throughout.

but for which rate of movement is governed by metabolic factors. One criterion for the existence of such a facilitated diffusion is that the rate of movement is affected by metabolic inhibitors. The influence of 3×10^{-4} M potassium cyanide (KCN) and of 5×10^{-5} M 2,4-dinitrophenol (DNP) upon the diffusion of DHO into sections was therefore measured. These concentrations inhibit net water uptake of sections essentially completely but do not kill the tissue. Figures 3 A and 3 B show that the two inhibitors have only a very slight depressant effect on permeability of sections to DHO.

Myers (9) has suggested that plasmolyzed cells may have a higher permeability to water than unplasmolyzed cells. An experiment was therefore done

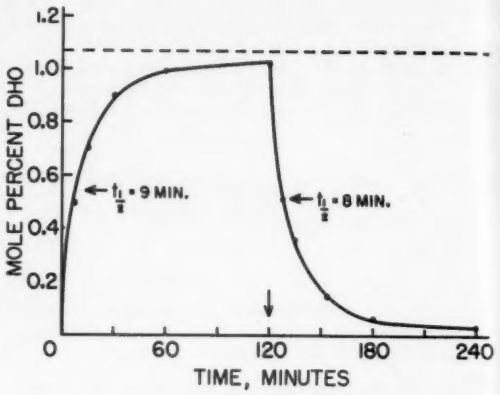

FIG. 2. Inward and outward diffusion of DHO in Avena coleoptile sections. External solution concentration indicated by horizontal line. Half-time indicated by arrows. 5 mg/l IAA and 0.0025 M maleate buffer pH 4.8 throughout.

to determine the rate of DHO movement into sections from a hypertonic mannitol solution. Tissue was equilibrated for 2 hours in 0.6 M mannitol before transfer to DHO-mannitol solutions. It was found that 0.6 M mannitol is without effect on the permeability of the coleoptile to DHO.

The experiments described thus far indicate that water moves into the Avena coleoptile by a simple diffusion process. The question may be raised whether the relative ineffectiveness of metabolic inhibitors on the diffusion of DHO in Avena sections is 1) because the plasma membrane offers essentially no resistance

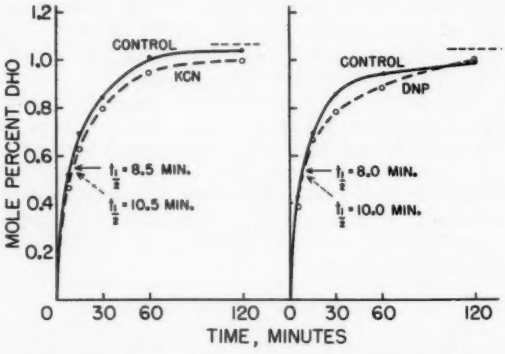

FIG. 3 A (*left*). Effect of 3×10^{-4} M KCN on inward diffusion of DHO into Avena coleoptile sections. External solution concentration indicated by horizontal line. Half-times indicated by arrows. 5 mg/l IAA and 0.0025 M maleate buffer pH 4.8 throughout.

FIG. 3 B (*right*). Effect of 5×10^{-5} M 2,4-DNP on inward diffusion of DHO into Avena coleoptile sections. External solution concentration indicated by horizontal line. Half-times indicated by arrows. 5 mg/l IAA and 0.0025 M maleate pH 4.8 throughout.

to water flow as compared to other structures, such as the cell wall, or 2) because the resistance of the intact membrane to water movement is not dependent on metabolism. If the first possibility were correct, then killing the cell should result in no appreciable increase in DHO permeability. If the second possibility were correct, the death of the cell should result in an increase in permeability, provided the living membrane offers as much or more resistance than the wall. Wartiovaara (11) has shown that D_2O diffuses out of living Tollypellopsis cells with a half time of one minute. When the cells are killed the half time is reduced to 1/3 minute. Thus in Tollypellopsis the living membranes offer more resistance to water flow than does the cell wall.

For the determination of the resistance to DHO movement contributed by the wall and by the membranes, Avena coleoptile sections were killed by a 4-hour pretreatment in 5×10^{-4} M DNP. After this treatment the cells of the section are irreversibly flaccid, their cytoplasm is extensively deranged as observed under the high power of the compound microscope and the sections are unable to grow in response to added auxin in the absence of DNP. The sections were then transferred to DHO solutions containing DNP in addition to the IAA and buffer. Figure 4 shows that the dead tissue possesses only a slightly increased permeability as compared to living tissue. The primary barrier to water movement in the Avena coleoptile thus appears to be a structure that remains relatively intact after death, presumably the cell wall. The cell membranes are even more highly permeable than the tissue values indicate. This explains in large measure why the various factors which influence net water uptake, such as external osmotic concentration, auxin and metabolic inhibitors have little or no effect on the absolute rate of movement of water into or out of this tissue.

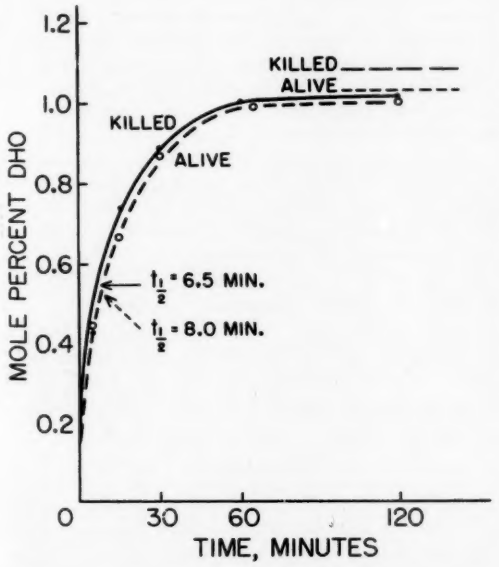

FIG. 4. Effect of killing by 5×10^{-4} M 2,4-DNP pretreatment on subsequent inward diffusion of DHO into Avena coleoptile sections. External solution concentrations indicated by horizontal lines. Half-times indicated by arrows. 5 mg/l IAA and 0.0025 M maleate pH 4.8 throughout.

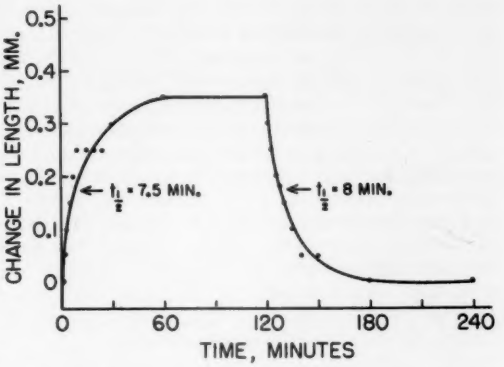

FIG. 5. Expansion and contraction of Avena coleoptile sections as a function of time when sections are transferred from 0.4 M mannitol to redistilled water and vice versa. Half-times indicated by arrows.

In order to compare the diffusion constants, measured by rate of exchange of labeled water, with the filtration constants determined osmotically, the latter were also determined with Avena section tissue. This was done by measuring the rate of expansion or contraction of sections when they were transferred from 0.4 M mannitol to distilled water and vice versa. Figure 5 shows that the time course of water uptake and loss obtained in this way is similar to that for the diffusion of DHO and that the half-times for change in length, 7.5 to 8 minutes, are of the same order of magnitude as the half-times for DHO diffusion. These results conflict with those of Buffel (2) who determined the filtration constants by a cartesian diver technique and obtained a half-time of 55 minutes. Buffel's method as applied to this determination is however subject to errors of interpretation as well as of technique, although it is quite adequate for the determination of D_2O diffusion constants.

The permeability constants of Avena section tissue to water are then essentially the same whether determined by rate of DHO diffusion or by osmotic means. It is of interest to compare these constants with those found for single plant cells. Huber and Höfler (4) have found half-times of plasmolysis varying from 20 seconds to nearly 6 minutes depending on the plant investigated.

Since the permeability data presented apply to the whole tissue and since the wall is the primary barrier to water movement, determinations of cell permeability using the osmotic method were made. It was found that the half time of protoplast shrinkage or

expansion in plasmolyzed subepidermal cells is 2 to 3 minutes and in cases in which the cell is close to a cut end of the tissue, the half-time is 1 minute or less. This indicates that an appreciable portion of the cell wall resistance to water movement may be located in the cutinized epidermal wall. Since deeper lying cells cannot be examined readily, no data are available on their half-times of expansion or contraction. These water movements occur across the cell wall as well as into the plasmolyzed protoplast. It may be that a part of the cause of the longer half-time for water diffusion into Avena tissue as compared to that into individual cells is merely the fact that water must traverse a series of cells as it moves from the external solution to the deeper lying interior cells.

Discussion

It has been found that various factors which have an important influence in governing net water uptake and metabolism in general, have little if any effect on the absolute rate of water movement in the Avena coleoptile section. Non-lethal concentrations of metabolic inhibitors, hypertonic conditions and auxin are included among these factors. The fact that death of the Avena section causes only a slight increase in water permeability, indicates that in this tissue the cell wall is the primary barrier to water movement. Kramer (6) and Brouwer (1), on the other hand, have shown that dead root systems show appreciably increased water conductivity as compared to living controls. Their observations were made by observing the rate of mass flow of water through the roots under the influence of artificial suction. This result indicates indirectly that the living membranes are more of a barrier to water movement than the cell wall in the case of roots.

There are few data in the literature which afford a direct comparison of living and dead cells with respect to true water permeability. Isotopic measurements are required for this purpose. Such an investigation has been carried out earlier with Tollypellopsis by Wartiovaara (11) who found that the water permeability of dead algal cells is three times that of living cells. Thus the living membranes are primary barriers to water movement in Tollypellopsis. It would be of interest to study the permeability of roots by the use of DHO since according to Brouwer (1) roots exhibit varying resistance to mass water movement depending on the conditions used.

A certain minimal degree of metabolism is required to maintain the integrity of the cell membranes. Metabolism, however, cannot directly govern tissue permeability to water in the Avena coleoptile, since the wall rather than the membrane is the limiting factor. The presence of the cell wall makes it difficult to assess accurately the role of metabolic processes in the regulation of membrane permeability.

Buffel (2) and Ketellapper (5) both obtained slightly shorter half-times of diffusion of D_2O with the cartesian diver technique than have been found in this investigation, probably due to their using slightly shorter sections. Ketellapper also found that as pH decreases below pH 4.5, permeability to D_2O increases. This increase may possibly be associated with injury to the tissue by the high acidity. The changes in water permeability found by Ketellapper are large as compared with those reported in this paper. It is possible that the cartesian diver technique used by Ketellapper although adequate for study of D_2O movement in the pH range above 4.5 with living sections (provided too long a period of exposure to high concentrations of D_2O is not used) is not adequate at low pH because of the flaccidity of the tissue.

Ordin, Applewhite and Bonner (10) have found that under optimal conditions (0.09 M sucrose, IAA) Avena sections of the kind used in this work elongate from 5.0 to 7.5 mm in about 13 hrs. The tissue then increases its net water content by half in 13 hours. This half-time is large compared with the half-time of water diffusion as measured in this investigation. The rate of water exchange is approximately 90 times the rate of net water movement. The high diffusion rate of water is consistent also with the fact reported earlier that the internal diffusion pressure deficit of Avena sections equals the external osmotic pressure under all conditions investigated (10). This high water permeability is consistent also with the early suggestion by Levitt (7, 8), that there is little likelihood of the occurrence of active water transport mechanisms in higher plants.

It has sometimes been found that isotopic and osmotic determinations of water permeability yield different values. Such discrepancies have given rise to speculations concerning the structure of the membranes. The absence of such a discrepancy for Avena coleoptile tissue makes it impossible to make such speculations about the nature or structure of the membrane or cell wall.

The finding that individual cells near the surface have a shorter half-time for water diffusion than the tissue as a whole suggests that in the case of Myers' (9) observations the difference between plasmolyzed and unplasmolyzed cell permeability is due to the time lag involved in water movement from the cell interior. A similar suggestion has been presented earlier by Levitt (8). In Myers' work cell permeability is apparently compared with tissue permeability rather than cell or tissue permeability under hypotonic solution conditions with that under hypertonic solution conditions.

Summary

Indoleacetic acid in optimal concentration has no effect on the diffusion of deuterium hydroxide solutions into Avena coleoptile sections.

The half-time of DHO diffusion into sections is 8 to 10 minutes. The inward and outward diffusion curves are identical in shape and half-times.

The state of turgor has no influence on the water permeability of this tissue.

Inhibitors such as 2,4-DNP and KCN in non-lethal

concentrations have only slight, if any, effect on permeability.

Killing of the tissue results in a very slight increase in permeability. The smallness of the increase indicates that the cell wall rather than the cell membranes is the primary barrier to water movement.

Determination of tissue permeability by osmotic means yields rates of water movement essentially identical to those obtained by the isotopic method. The half-time of protoplast expansion or contraction of individual cells located near the surface is however 2 to 3 minutes and in cases in which the cells are very near to cut tissue edges, 1 minute or less. Cell permeability of Avena coleoptile section tissue is thus greater than the permeability of the tissue as a whole.

The DHO analyses were carried out by the analytical service department of the Consolidated Engineering Corporation. We wish to thank Mr. William Bailey and Mr. Jack Walker for their helpful counsel.

Literature Cited

1. Brouwer, R. The regulating influence of transpiration and suction tension on the water and salt uptake by the roots of intact *Vicia Faba* plants. Acta Bot. Néerl. 3: 264–312. 1954.
2. Buffel, K. New techniques for comparative permeability studies on the oat coleoptile with reference to the mechanism of auxin action. Mededel. Koninkl. Vlaamse Acad. Wetenschap., België 14: No. 7. 1952.
3. Danielli, J. F. Morphological and molecular aspects of active transport. Symposia Soc. Exptl. Biol. 8: 502–516. 1954.
4. Huber, B. and Höfler, K. Die Wasserpermeabilität des Protoplasmas. Jahrb. wiss. Bot. 73: 351–511. 1930.
5. Ketellapper, H. J. The mechanism of the action of indole-3-acetic acid on the water absorption by Avena coleoptile sections. Acta Bot. Néerl. 2: 388–444. 1953.
6. Kramer, P. J. The intake of water through dead root systems and its relation to the problem of absorption by transpiring plants. Amer. Jour. Bot. 20: 481–492. 1933.
7. Levitt, J. The thermodynamics of active (non-osmotic) water absorption. Plant Physiol. 22: 514–525. 1947.
8. Levitt, J. Further remarks on the thermodynamics of active (non-osmotic) water absorption. Physiol. Plantarum 6: 240–252. 1953.
9. Myers, G. M. P. The water permeability of unplasmolyzed tissues. Jour. Exptl. Bot. 2: 129–144. 1951.
10. Ordin, L., Appelwhite, T. H., and Bonner, J. Auxin-induced water uptake by Avena coleoptile sections. Plant Physiol. 31: 44–53. 1956.
11. Wartiovaara, V. The permeability of Tolypellopsis cells for heavy water and methyl alcohol. Acta Bot. Fenn. 34: 1–22. 1944.
12. Washburn, H. W., Berry, C. E., and Hall, L. G. Measurement of deuterium oxide concentration in water samples by the mass spectrometer. Anal. Chem. 25: 130–134. 1953.

A STATISTICAL EVALUATION OF SOME OF THE FACTORS RESPONSIBLE FOR THE FLOW OF SAP FROM THE SUGAR MAPLE[1,2]

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No adequate explanation has yet been proposed for the mechanism responsible for vessel sap pressures and sap flows from wounded stems in several species of Acer and some other genera during the dormant period. It is generally agreed that the pressures and flows are temperature-induced. Clark (1, 2) and Jones (5, 6) many years ago made extensive observations on soil, air and stem temperatures and the sap flows from trees in the field. More recently, similar observations on excised stems have been made by Stevens and Eggert (8) and on both stems and stumps by Johnson (4). On the basis of these field observations, a number of authors have proposed and discussed possible mechanisms responsible for the pressures and flows (1, 2, 4, 5, 8 and 9). Most of the observations have been made in the field, but Marvin and Greene (7) have reported on a series of experiments on excised stems under conditions of controlled temperature in the laboratory. Some of these investigators have observed that sap pressures and flows occur only following a rise in the temperature of the stem tissues and also that the pressures and rates of flow decrease when the temperatures fall.

Temperature and flow rate or flow volume data have not heretofore been given a critical analysis to evaluate the possible relationship between these variables. In the experiments reported here temperature and flow rate data have been collected for several trees for four seasons. A multiple regression analysis of these data has demonstrated a quantitative relationship between the daily total volume of sap flow and four independent variables derived from the temperature and flow data.

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Materials and Methods

The data presented here were collected during the months of March and April for the years of 1950, 1951, 1952 and 1953 at the Proctor Maple Research Farm of the College of Agriculture, University of Vermont. The temperature and sap flow data were recorded from mature trees of *Acer saccharum* Marsh. Using copper-constantan thermocouples, a continuous record through each season was made of air temperatures in the shade at 6 ft (1.8 m) and at 30 ft (9.1 m) in the crown. Temperatures of the xylem were recorded 6 cm inside the stem surface as well as temperatures of the inner bark. Twig temperatures were obtained by inserting the thermocouple into the cut end of a twig 6 mm in diameter, 30 ft from the ground in the center of the crown. The thermocouple junctions were inserted at least 5 cm into the tissues and were sealed with plastic wood. These and other temperatures were recorded by two 16-point Leeds and Northrup Micromax recording potentiometers.

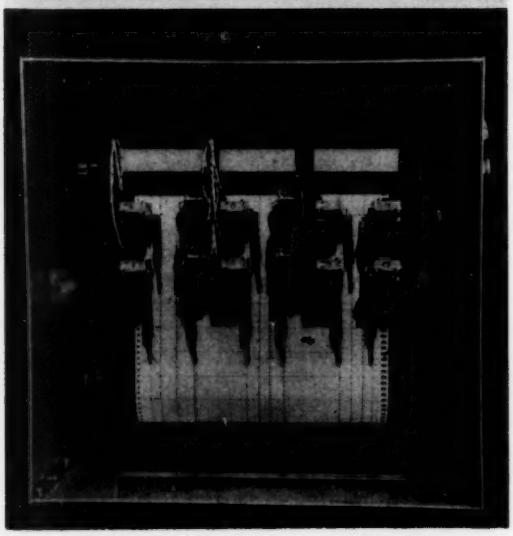

Fig. 1 (*above*). Tipping bucket measuring element.
Fig. 2 (*below*). Multiple recorder for the tipping buckets.

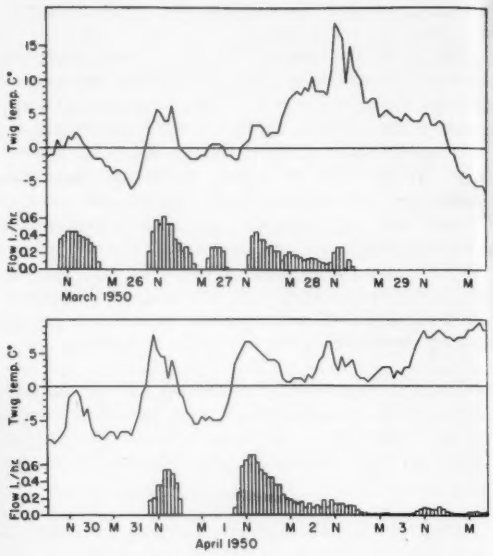

Fig. 3. Twig temperatures and sap flow rates for tree 500 for nine days plotted at hourly intervals.

Sap flow rates and volumes were continuously recorded by devices constructed for the purpose, shown in figures 1 and 2. The sap flowed from a standard tap hole (7/16 in × 2 in) through a rubber tube to a tipping bucket unit mounted on a large reservoir. The measuring unit was similar to that of a rain gauge. The capacity of each bucket was 15 ml. When the bucket tipped to empty, it momentarily closed a magnetically operated switch which sent an impulse to the recorder. The recorder consisted of a Leeds and Northrup chart frame to which 12 relays with pens mounted on the armatures were attached. The pens recorded on a chart which moved at the same rate of speed as the potentiometer charts. The rates of flow could then be compared with the temperatures for any given time or period of time.

Results

The several temperatures and rates of sap flow were plotted at hourly intervals for each of the four seasons for three trees. A careful study of these graphs indicated that twig temperatures correlated best with flow rates. Frequently rapid flow rates were observed when there had been no change in the xylem and inner bark temperatures, although the temperature of the twig had increased. However, on some days with bright sun and a cold wind, the xylem and inner bark temperatures may have risen as much as 10° C, whereas a change of less than 1° C above 0° C

was observed in the twig temperature and no flow occurred. Several trees were decapitated 20 ft (6.1 m) above the ground but below the first branches, and the cut surface was sealed with a rubber-faced cap. The rates and volumes of flow were much reduced following the treatment and were found to correlate with changes in the temperatures of the inner bark and xylem. It was concluded from these observations that twig temperatures correlated best with the flow rates and volumes, and were therefore used in the statistical analysis.

Twig temperatures and flow rates for one tree are plotted for hourly intervals in figure 3. Two characteristic temperature and flow relationships are apparent. When a flow occurs it follows a rise in temperature above 0° C and a decrease in temperature is followed by a decrease in the rate of flow and, in some cases, by a cessation of flow. It may also be observed that the rate of flow is not proportional to the magnitude of the rise in temperature, as, for example, in the flows recorded on March 26 and 27, April 1 and 3.

Experiments on stem sections previously reported (7) and the experiments of Stevens and Eggert (8) have demonstrated that during the cooling period preceding a flow, stems absorbed water or an aqueous solution. If the stems were not permitted to absorb, the flow following a cooling period was greatly reduced or did not occur at all. These observations suggested that an important relationship might be found between preceding temperatures and flows. A period of freezing temperatures (7) is not a necessary prerequisite for a flow. Figure 3 (March 28, April 2 and 3) shows that a rising temperature from a previous low above 0° C increases the rate of flow. However, it is apparent in figure 3 that when the preceding temperature was below 0° C the flow was greater both in rate and volume than when the lowest temperatures between the flows were above 0° C. On March 29 temperature rises comparable to those of March 27 did not cause a flow, and on April 2 and 3 temperature rises had only slight effects on the rate of flow.

An attempt has been made to formulate these relationships by the methods of regression analysis outlined by Ezekiel (3). Daily volumes of flow, Y, were related to varying numbers of independent variables, X_i, variously defined, and in various functional relationships, such as multiple linear, exponential and polynomial. In each trial, a least-squares solution of the regression was found, values of the predicted flow, Y', calculated, and the coefficient of multiple correlation, R, of Y and Y' values used to assess the goodness of fit. After a number of trials, the best fit of the flow data was obtained from a multiple linear regression on four independent variables, three of which were derived from the temperature data, and one from the flow data, as described below. It should be realized that the success of an analysis such as this is greatly dependent upon the insight and ingenuity of the analyst, and we have no doubt that it would be possible to improve considerably upon the prediction of flow rates which our analysis permits.

The equation finally adopted had the form

$$Y' = C(b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4)$$

where Y' is the predicted flow rate, b_i are constants, and X_i and C, the independent variables, as defined below. An example of observed flow rates, Y, appears in table I. X_1 is the number of hours during which twig temperatures were below 0° C, cumulated for the 24-hr period from 6:00 A.M. to 5:00 A.M. inclusive, preceding the flow in question. It may be termed the "conditioning" factor, and was suggested by the experiments of Stevens and Eggert (8), and Marvin and Greene (7) referred to above. For many years,

TABLE I

DAILY SAP FLOW (Y), AND THE INDEPENDENT VARIABLES (X_1 TO X_4) TO WHICH IT IS RELATED FOR TREE 500 IN 1950

$Y = -3.491 + 2.135X_1 + 0.340X_2 + 0.026X_3 + 0.412X_4$

DATE	Y	X_1	X_2	X_3	X_4
Mar. 20	8.8	0	0.0	74	0
21	13.2	13	8.8	133	1
22	36.8	11	13.2	189	2
23	9.4	5	36.8	60	3
24	10.7	0	0.0	0	0
25	43.8	24	10.7	15	5
26	65.4	18	43.8	65	6
27	47.8	16	65.4	147	7
28	17.5	6	47.8	97	8
29	0.0	0	17.5	7	9
30	0.0	0	0.0	0	0
31	36.3	24	0.0	60	11
Apr. 1	84.6	16	36.3	127	12
2	19.6	3	84.6	95	13
3	11.2	0	19.6	275	14
4	22.7	0	11.2	311	15
5	0.0	0	22.7	26	16
6	0.0	0	0.0	0	0
7	32.2	24	0.0	38	18
8	73.8	16	32.2	75	19
9	0.6	0	0.0	0	0
10	50.0	22	0.6	28	21
11	149.6	17	50.0	124	22
12	34.1	3	149.6	19	23
13	0.0	0	0.0	0	0
14	149.3	72	0.0	93	25
15	121.7	14	149.3	59	26
16	64.8	8	121.7	288	27
17	8.2	1	64.8	217	28
18	12.1	0	8.2	222	29

farmers have observed that a storm with wet snow that adheres to the trees precedes the largest flow. Criteria for this weather condition were established from field notes and the temperature data and, where these criteria applied, the conditioning factor (X_1) was increased by a factor of 3. The factor was chosen after preliminary analyses showed that flows on days characterized by wet snows were exceptionally large. This condition occurred five times in the four years—for example, April 14, 1950, table I.

The temperatures initiating a flow and during a flow were quantized by cumulating degree-hours above 0° C, or the lowest temperature for the previous 24-hr period. They were tabulated for the day of each flow

Table II

Total Volume (Liters) of Sap Yielded by Three Trees in Four Years

Year	Tree		
	457	499	500
1950	54	50	101
1951	57	98	129
1952	26	56	69
1953	..	52	82

For difference between trees 457 and 499 $t_3 = 2.28$; $P < 0.25$.
For difference between trees 457 and 500 $t_3 = 5.95$; $P < 0.05$.
For difference between trees 499 and 500 $t_3 = 4.02$; $P < 0.05$.

from 6:00 A.M. to 5:00 A.M. An example of these data appears in table I as X_3. An examination of the seasonal pattern of twig temperatures and flow rates indicated that in some years the mechanism was more responsive to temperature changes late in the season than earlier. The X_4 term, simply the serial number of the day of the flow, represents this seasonal effect, and in these analyses the use of this term improved the relationship. Its biological significance is not known.

It seemed reasonable to assume that the magnitude of a flow might be related to that of the preceding one. It was supposed that a large flow would deplete the system and tend to make the succeeding flow smaller. Accordingly, the flow volumes for the preceding 24 hrs were tabulated and are the X_2 term.

Table III

Coefficients for the Multiple Regression * for Each Tree for Each Year and a Summary for Each Tree for the Years Combined

Tree	b_0	b_1	b_2	b_3	b_4
		1950			
457	−2.388	+1.034	+0.168	+0.002	+0.699
499	−0.631	+1.158	+0.286	+0.008	+0.115
500	−3.491	+2.135	+0.340	+0.026	+0.412
		1951			
457	+2.403	+0.935	+0.098	+0.166	−0.352
499	+4.447	+2.019	+0.109	+0.290	−0.810
500	+5.933	+1.920	+0.068	+0.328	−0.479
		1952			
457	−4.585	+1.431	+0.310	−0.005	+0.314
499	−2.521	+2.725	+0.151	−0.006	+0.463
500	−5.219	+3.026	+0.127	+0.002	+0.774
		1953			
499	+5.307	+2.212	−0.014	+0.043	−0.297
500	+16.225	+3.131	−0.014	+0.059	−0.749
		Combined			
457	−1.106	+1.134	+0.295	+0.055	−0.050
499	+2.741	+1.778	+0.161	+0.079	−0.282
500	+3.780	+2.387	+0.171	+0.100	−0.210

* $Y' = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4$.

The flow data Y and X_2 are in arbitrary units and may be converted into ml by multiplying each daily flow value by 90. The terms X_1 and X_3 are in Fahrenheit units, for convenience in calculating. They may be converted to Centigrade degree-hours by multiplying by 0.56. In nature, the temperature may rise above 0° C although neither high enough nor long enough to thaw the tissues and permit a flow. Therefore, the convention was adopted that when X_3 was less than 5, C is equal to zero, and otherwise has the value of 1.0.

The yields of sap for each tree for each year are shown in table II, as well as the statistical evidence that there are significant differences in the yields between the trees. The coefficients of the multiple regressions for each tree for each year, and for each tree for the years combined, are shown in table III. In comparing the yields of the three trees and the b-values, no consistent pattern is apparent from year to year. However, when the years are combined, the b_1 values are ranked in the same order as the total yield from the trees. In contrast, the b_3 values are essentially the same. The b_4 values are in some years positive and in some years negative, negative when the seasonal effect is large at first and diminishes during the season, positive when the seasonal effect increases during the season. Contrary to the supposition on which X_2 was defined, the coefficients b_2 are positive, except in 1953. That is to say, large flows tend to occur on successive days, and there is no evi-

Table IV

Coefficients of Separate Determination (d), Coefficients of Multiple Correlation (R^2) and Variance Ratios (F = Regression Mean Square/Residual Mean Square) for Multiple Regressions Fitted to the Data for Each Tree Each Year and the Years Combined

Tree	ΣY	d_1	d_2	d_3	d_4	R^2	F	Values of F for P = 0.001
				1950				
457	600.8	.341	.063	.009	.125	.538	9.42	6.4
499	555.6	.439	.067	.002	.017	.525	8.85	6.4
500	1124.2	.477	.105	.006	.044	.632	14.90	6.4
				1951				
457	633.1	.198	.009	.257	.002	.466	8.69	6.1
499	1086.4	.296	.011	.247	.015	.568	13.70	6.1
500	1433.6	.229	.003	.276	−.006	.503	10.28	6.1
				1952				
457	289.3	.438	.107	−.002	−.005	.538	10.25	6.3
499	624.8	.528	.040	.000	−.016	.551	10.80	6.3
500	771.4	.465	.029	.001	.011	.505	8.84	6.3
				1953				
499	582.3	.733	.000	.028	.013	.774	46.80	5.3
500	908.2	.727	.000	.024	.050	.801	58.75	5.3
				Combined				
457	1523.2	.335	.079	.049	−.003	.459	21.74	5.0
499	2849.1	.406	.022	.045	.003	.476	32.80	5.0
500	4237.4	.454	.025	.048	−.001	.527	40.42	5.0

dence for the supposed depletion. Nevertheless, this term contributes somewhat to the regression and has been retained.

The numerical values of the coefficients of any one equation cannot be compared, since they depend on the units chosen to represent the variables. The importance of the separate terms of the regression can, however, be compared by use of the coefficients of separate determination (Ezekiel, 3), and these are shown in table IV, for the regressions of table III, together with multiple correlation coefficients squared, R^2, and ratios of residual to regression variance, F. It is apparent that the "conditioning" factor, X_1, has the greatest value in prediction of flows. This is apparent in the combined values.

Discussion

For many years it has been evident that a relationship exists between tissue temperatures and the sap flow in maples. There have been, however, no published data attempting to demonstrate a quantitative relationship between differing temperatures and the volumes of sap flow. It has been possible in this analysis to account for a statistically significant portion of the flow for each of three trees for four years.

Flow has been visualized in this analysis as dependent upon four independent variables derived from the temperature data. The two most interesting independent variables are the temperature terms X_1 and X_3. With the exception of 1951, the X_1 term, the conditioning factor, makes the largest single contribution. The coefficient of separate determination d_1 indicates that X_1 accounts for the major portion of the flow (table IV). This is shown when the years are combined, as well as in individual years. It would appear, therefore, that variations in flow are more closely related to the preceding temperatures than to the temperatures at the time of the flow. Data are not at hand to support adequately an hypothesis for the mechanism of flow, but these particular data do suggest that the X_3 term, that of the temperatures prevailing on the day of the flow, has an indirect rather than a direct thermal effect on the tissues producing the flow. This strongly suggests a trigger mechanism. The X_2 and X_4 terms do not show a consistent pattern through the four years and possibly could have been left out of the analysis without serious consequences.

Although the four independent variables considered here account for a significant portion of the flows observed, other factors undoubtedly influence the volume of flow. Soil temperature and soil moisture are probably both important, during the sap flow season and the preceding months. The rainfall and temperatures throughout the preceding months may also have an effect. Data on these factors would be required to establish such relationships.

Summary

Hourly data on the flow of sap from three sugar maple trees, *Acer saccharum* Marsh., during March and April of 1950, 1951, 1952 and 1953 have been obtained from an automatic recording device. Wood, bark, twig and air temperatures were recorded simultaneously for the same trees. Preliminary study of the data showed that the flows were more closely related to twig temperatures than to the other temperatures recorded, and twig temperatures have been used in a multiple regression analysis. A significant portion of the daily flow can be accounted for by a multiple linear regression of flow rate on 1) the number of hours during the day preceding the flow, when twig temperature was below 0° C; 2) the total volume of flow on the preceding day; 3) the number of degree-hours above a previous low, cumulated for the day of the flow; and 4) the serial number of the day of the flow. Of these 1) is of most importance, indicating that preceding temperatures are more important than current temperatures in determining the amount of a flow. The fact that flows characteristically begin after a rapid temperature rise above freezing suggests a "triggering" effect of temperature.

Literature Cited

1. Clark, W. S. The circulation of sap in plants. Ann. Rept. Secy. of Agr., Massachusetts 21st (1874): 159–205. 1874.
2. Clark, W. S. The flow of sap and the power of plant growth. Ann. Rept. Massachusetts Agricultural College 12th (1875): 18–53. 1875.
3. Ezekiel, M. Methods of Correlation Analysis. 2nd ed. Pp. 1–531. Wiley, New York. 1941.
4. Johnson, L. P. V. Physiological studies on sap flow in the sugar maple, *Acer saccharum* Marsh. Canadian Jour. Research C 23: 192–197. 1945.
5. Jones, C. H., Edson, A. W., and Morse, W. J. The maple sap flow. Agr. Expt. Sta., Vermont, Bull. 103. 1903.
6. Jones, C. H. and Bradlee, J. L. The carbohydrate contents of the maple tree. Agr. Expt. Sta., Vermont, Bull. 358. 1933.
7. Marvin, J. W. and Greene, M. T. Temperature-induced sap flow in excised stems of Acer. Plant Physiol. 26: 565–580. 1951.
8. Stevens, C. L. and Eggert, R. L. Observations on the causes of the flow of sap in red maple. Plant Physiol. 20: 636–648. 1945.
9. Wiegand, K. M. Pressure and flow of sap in the maple. Amer. Naturalist 40: 409–453. 1906.

THE SALT RESPIRATION AND PHOSPHATE CONTENTS OF BARLEY ROOTS[1,2]

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Phosphorylative coupling reactions are capable of controlling both respiration and fermentation by adjusting amounts of phosphate acceptor and inorganic phosphate (19, 38). Salt-induced respiration which is associated with endergonic salt accumulation (23) may also be controlled by phosphorylative coupling reactions. While respiration generally was increased by DNP[4] which can release respiration from the control of phosphorylation, salt accumulation was decreased or completely abolished (29, 37). A direct or indirect participation of phosphorylative coupling reactions in salt accumulation might be inferred.

On the other hand, salt respiration may result from an effect of inorganic ions themselves, i.e., the ionic milieu may influence the rate of respiratory activity. Inorganic anions may have a "coenzyme" effect by balancing a temporary charge difference produced in the separation of electrons from substrates and their subsequent transfer through the cytochrome system (22). It is difficult to formulate a mechanism to explain how ions can facilitate stoichiometric separation of charges unless ion-pair formation occurs. A salt-cytochrome complex may facilitate electron transfers similarly to those complexes proposed for the bridge transfer mechanism of electron transfer in the oxidation-reduction of metallic ions in solution (43).

The possible salt influences upon the cytochrome system and the phosphorylative coupling system in the stimulation of respiration are not necessarily mutually exclusive effects. It is now well established that oxidative phosphorylations occur in the cytochrome system (20, 26). It is pertinent that some DNP effects on phosphorylations may be associated with its possible incorporation into the oxidation-reduction complex (6). There is no evidence whether DNP prevents salt accumulation because it prevents net production of energy-rich phosphate bonds or whether it prevents salt accumulation and energy production independently of each other.

This investigation was to obtain some evidence about the nature of salt respiration by determining the correlation of respiration of barley roots, treated in various ways, with the root contents of inorganic and organic phosphate. It was hoped to find whether the salt stimulation of oxygen uptake has effects on the phosphate contents similar to those often associated with the DNP stimulation of oxygen uptake.

METHODS AND MATERIALS

PREPARATION OF BARLEY: Roots of 5-day-old barley seedlings (*Hordeum vulgare*, vars. Atlas 46 and Moore) were used as the experimental material. The seedlings were prepared according to a modification (13) of the procedure of Machlis (24). The roots after 5 days growth were about 20 cm long and possessed few root hairs.

RESPIRATORY MEASUREMENT: Respiration was determined by the direct manometric method of Warburg at 25° C. Usually 150 segments, either 5 mm or 10 mm long, per 2.50 ml of solution were used. The root segments were initially in 2.00 ml of distilled water. After about 100 minutes when the respiratory drift of recently harvested root segments became negligible, 0.50 ml of test solution was tipped into the main chamber of the Warburg vessel. Oxygen uptakes were computed from time of tipping. The data presented for preliminary salt respiration and DNP studies are the averages of at least three but mostly five separate experiments at each point.

Different DNP effects can result from variation of pH, concentration and temperature, and also from times of treatment. An extreme DNP effect on different root regions is illustrated by the respiration of Moore roots treated with 2×10^{-4} M DNP. The total oxygen uptake of the 0 to 5 mm root regions was increased to 183 % of the water controls but respiration was reduced to 53 % of the water controls for the 5 to 10 mm region (where the root tip is the zero point). Because of the difference in sensitivity, different concentrations of DNP were applied to the different root regions for the experiments on correlations of respiration with phosphate contents. These concentrations were known to bring about maximal increases in respiration for the particular regions. The particular effects reported for the preliminary studies were based upon a standard treatment of thirty minutes. DNP treatment times for the correlative tests with phosphates were 15, 30 and 60 minutes.

PHOSPHATE ANALYSES: Inorganic phosphate and total phosphate of the cold-TCA-extracts of the roots and the total phosphate of the cold-TCA-insoluble phosphate were determined directly by a modification of the Lowry-Lopez method (21). TCA-soluble organic phosphate was calculated by the difference be-

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[4] Abbreviations employed in this paper are as follows: DNP, 2,4-dinitrophenol; TCA, trichloroacetic acid; ATP, adenosinetriphosphate; ADP, adenosinediphosphate; DNAP, desoxyribose nucleic acid phosphorus; RNAP, ribose nucleic acid phosphorus.

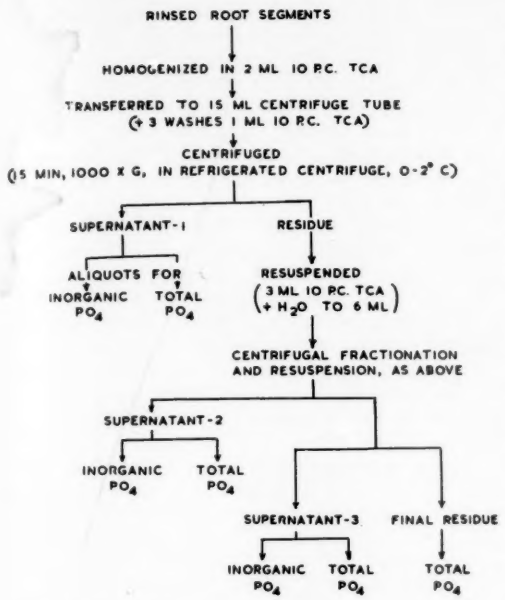

RINSED ROOT SEGMENTS
HOMOGENIZED IN 2 ML 10 P.C. TCA
TRANSFERRED TO 15 ML CENTRIFUGE TUBE (+ 3 WASHES 1 ML 10 P.C. TCA)
CENTRIFUGED (15 MIN, 1000 X G, IN REFRIGERATED CENTRIFUGE, 0-2° C)
SUPERNATANT-1
RESIDUE
ALIQUOTS FOR INORGANIC PO4 TOTAL PO4
RESUSPENDED (3 ML 10 P.C. TCA + H2O TO 6 ML)
CENTRIFUGAL FRACTIONATION AND RESUSPENSION, AS ABOVE
SUPERNATANT-2
INORGANIC PO4
TOTAL PO4
SUPERNATANT-3
FINAL RESIDUE
INORGANIC PO4
TOTAL PO4
TOTAL PO4

FIG. 1. Extraction and fractionation procedure for phosphate contents in barley root segments.

tween total cold-TCA-soluble phosphate and inorganic phosphate.

One hundred and fifty segments of 10 mm length, weighing about 200 mg fresh weight, were removed from the Warburg vessels at 0, 15, 30 and 60 minutes after adding solutions containing KBr, DNP, KBr + DNP, or water. The segments were rinsed with ice-chilled distilled water on a Hirsch funnel. The segments were homogenized in 2 ml of 10 % TCA with a Potter-Elvehjem homogenizer chilled in a salt-ice mixture. The time from removal of the Warburg vessels from the temperature bath to completion of the homogenization was less than 2.5 minutes. Further extraction and fractionation of the TCA homogenate followed the procedure outlined in figure 1. All materials were ice-chilled.

In the inorganic phosphate analyses, 2 ml aliquots from the TCA supernatants were added to 2 ml of sodium acetate reagent plus 4 ml acetate buffer reagent. One ml of ascorbic acid reagent was added. The preceding steps were carried out with ice-chilled reagents and apparatus. After color development at room temperature for 10 minutes following the addition of 1 ml molybdic acid reagent, the light absorption of the solution at 660 mμ was compared with that of a standard series containing up to 0.70 micromoles phosphate per tube. A model DU Beckman spectrophotometer was employed for the analyses.

In the total phosphate analyses 0.5-ml aliquots of 7 *N* sulfuric acid as required were added to 1-ml aliquots of the TCA supernatants and to the entire cold-TCA-insoluble residues. The mixtures were digested over a microburner until clear, then decolorized with reagent-grade 30 % H_2O_2. The digests were diluted to 8-ml and 2-ml aliquots from the diluted digests were added to 3 ml sodium acetate reagent plus 4 ml acetate buffer reagent. Subsequent steps were identical to those used for the inorganic phosphate analysis.

Compositions of the reagents for the modified Lowry-Lopez method were as follows: the molybdic acid reagent contained ammonium molybdate one gm in 100 ml 0.05 *N* sulfuric acid; the ascorbic acid reagent contained one gram ascorbic acid in 100 ml water; the sodium acetate reagent was 1 M; and the acetate buffer (pH 4) was composed of equal volumes of 1 *N* acetic acid and 0.25 M sodium acetate.

The cold-TCA-insoluble phosphate fraction probably contained mainly nucleic acid and the phospholipid phosphates. The cold-TCA-soluble organic phosphate fraction probably contained the low molecular weight phosphate compounds, e.g., phytic acid, sugar phosphates, phosphopyridine nucleotides, etc.

RESULTS

PRELIMINARY STUDIES ON ROOT RESPIRATION, *Effects of KBr:* The root regions were examined for gradients to characterize the root behavior and to facilitate the correlative studies on phosphate contents and respiration.

The increases in respiration on addition of a particular KBr concentration to the apical segments, i.e., the salt respiration, were smaller than those for basal segments (figs 2 and 3); but the total respiration was greater in the apex (25).

For both Moore and Atlas 46 barley only a low concentration of KBr was necessary to induce a near-maximal salt respiration (fig 4). For later experiments 0.05 KBr was chosen as a convenient concentration to induce a marked salt respiration.

Effects of DNP: The concentration of DNP for maximal oxygen uptake was substantially the same as that for maximal stimulations of various other tissues (3, 28). For a 30-minute treatment this generally oc-

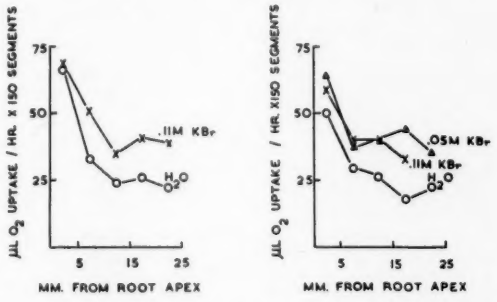


FIG. 2 (*left*). Root gradient of oxygen uptake by Atlas 46 barley after 30 minutes in water and KBr (0.5 cm segments).

FIG. 3 (*right*). Root gradient of oxygen uptake by Moore barley after 30 minutes in water and KBr (0.5 cm segments).

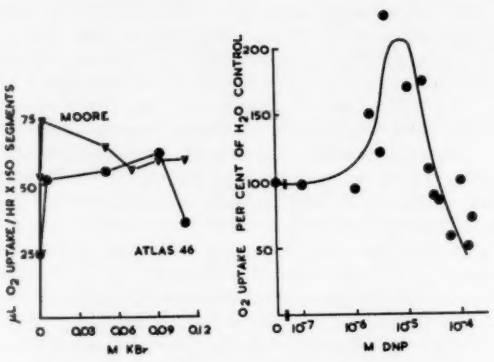


FIG. 4 (*left*). Oxygen uptake by Atlas 46 roots (10 to 15 mm region) and Moore roots (0 to 5 mm region) after 30 minutes in water and KBr.

FIG. 5 (*right*). Oxygen uptake by Moore roots after 30 minutes in DNP (averages of all regions tested).

curred with DNP between 5×10^{-6} to 5×10^{-5} M (fig 5). As pointed out earlier, the type and degree of response also depended upon the root region.

Respiration of the same root regions was sometimes affected differently by the same DNP treatment. For DNP lower than 3×10^{-6} M inhibitions never resulted for any root region, whereas for DNP greater than 10^{-5} M inhibitions often occurred. The usual effect was to increase respiration between these DNP concentrations. As no buffers were added, fluctuation of solution pH probably induced variable effects reflecting the effectiveness of DNP reaching its sites of action.

PHOSPHATE CONTENTS AND OXYGEN UPTAKES, *Initial Phosphate Contents:* Table I presents the initial phosphate contents of the Moore barley used for correlation studies between oxygen uptake rates and phosphate contents. Phosphate contents of the root segments after various treatments are not given directly but in terms of correlations. Changes in phosphate contents were small and of no discernible pattern in themselves. Loss of phosphate from the roots occurred in all treatments involving DNP. However, the losses (about 1 % in 60 min of the total initial content) could not be specifically related to change in any phosphate fraction.

A significant positive correlation was found between TCA-insoluble phosphate and TCA-soluble organic phosphate (table II). No other correlation was found between the various phosphate fractions.

Respiration of Roots in Water: There was a high positive correlation between the respiration rate of roots in water and the cold-TCA-insoluble phosphate per segments (fig 6 and table III). Respiration was not found to be correlated with any other phosphate fraction.

Figure 8 presents a transformation of the linear lines of best fit of respiration rate per segments (fig 7) into specific respiratory activities (oxygen uptake per hour per micromole cold-TCA-insoluble phosphate) plotted against TCA-insoluble phosphate content. The cluster of scatter points for roots of high phosphate content were from the 0 to 10 mm apical segments in all cases (figs 7 and 8). The curves are not extrapolated beyond the extreme values of TCA-insoluble phosphate content found for the 0 to 30 mm root region. Thus these transformed curves represent the gradient of specific respiratory activity only between the 0 to 30 mm region. It appears that the specific respiratory activity of regions more basal would not have changed much since respiration (figs 2 and 3) and phosphate content (table I) had reached or approached constant values per regions.

For roots in water, in contrast to the oxygen uptake rate per segments (figs 2 and 3) the specific respiratory activities were greater for the root regions

TABLE I

PHOSPHATE CONTENTS OF MOORE BARLEY ROOT SEGMENTS BEFORE TREATMENT WITH WATER, KBr, DNP, AND KBr + DNP

TREATMENT	AGE	REGION FROM APEX	FRESH WT	COLD-TCA-INSOLUBLE	COLD-TCA-SOLUBLE	
					ORGANIC	INORGANIC
	days	*mm*	*mg/150 segments*	*micromoles PO_4/150 segments*		
KBr	5	0–10	165 ± 0.9 *	2.06	2.88	1.23
DNP	5	0–10	186 ± 0.6	1.83	2.92	0.89
KBr + DNP	5	0–10	183 ± 0.6	1.82	2.35	0.74
KBr	5+2 **	10–20	207 ± 1.3	0.91	2.30	1.03
DNP	5+1	10–20	195 ± 0.6	0.67	2.13	0.52
KBr + DNP	5+2	10–20	192 ± 0.5	0.70	1.51	1.13
KBr	5+3	20–30	219 ± 1.7	0.57	2.03	0.85
DNP	5+3	20–30	213 ± 1.0	0.67	2.33	0.84
KBr + DNP	5+4	20–30	210 ± 1.3	0.76	1.49	0.47

* Standard error of the mean, corrected for number of cases, seven.

** Roots were harvested from 5-day-old seedlings and the segments stored for two days at 7° C in distilled water. The segments from different root regions for each treatment, e.g., all KBr-treated segments, are from the same plants but stored for different times after harvest.

TABLE II

CORRELATION * BETWEEN THE PHOSPHATE CONTENTS OF MOORE BARLEY ROOT SEGMENTS BEFORE TREATMENT WITH WATER, KBr, DNP, AND KBr + DNP

MEASUREMENT	PHOSPHATE FRACTIONS CORRELATIONS					
	INSOLUBLE–ORGANIC		INSOLUBLE–INORGANIC		ORGANIC–INORGANIC	
	(X)	(Y)	(X)	(Y)	(X)	(Y)
Coeff of correlation, r	0.67 ± 0.18 **		0.23 ± 0.31		0.24 ± 0.31	
Coeff of regression, b †	0.61 ± 0.28		0.18 ± 0.73		0.19 ± 0.59	
Intercept, a †	1.53		0.66		0.44	
Standard error of estimate (X), micromoles PO_4/150 seg	0.43		0.56		0.46	
Standard error of estimate (Y), micromoles PO_4/150 seg	0.35		0.24		0.23	
Number of cases	9		9		9	

* All values corrected for number of cases.
** Significant at P = 0.05.
† Calculations for the line of regression Y = a + bX, by the method of least squares.

located progressively more distant from the root apex (fig 8, water control).

Respiration of Roots in KBr Solution: The correlation of specific respiratory activity with insoluble phosphate content (fig 8) illustrates in another form the relationship shown in figures 2 and 3. The basal regions containing lower amounts of cold-TCA-insoluble phosphate had not only a greater net salt respiration rate per segments but also a greater net salt respiration rate per metabolizing unit as estimated by TCA-insoluble phosphate.

The slope of the regression curve for the correlation between respiration rate per segments and the TCA-insoluble phosphate content was significantly different from the water control and the DNP-treated roots (table III). Correlation between respiration rate and insoluble phosphate remained high and significant.

The respiration rate of the KBr-treated roots was not significantly correlated with inorganic or organic phosphate.

Respiration of Roots in DNP Solution: The net DNP effect on specific respiratory activity was essentially independent of the amount of respiring materials, thus independent of the root regions tested (fig 8). It is emphasized that this is quite different from the DNP effects viewed from respiration rate per segments (fig 7). DNP failed to abolish the differential specific respiratory activity gradient along the root axis (fig 8, DNP treatment compared with the water control).

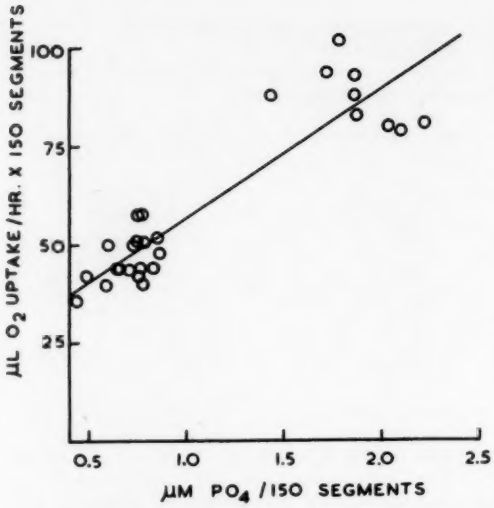
Although a negative correlation between respiration rate per DNP-treated segments with organic phosphate was found (r = − 0.22 ± 0.40) it was not significant. However, the correlation was significantly different (P = 0.01) in the direction expected from the correlation for the water control (r = + 0.34 ± 0.17). Neither was the correlation of respiration with inorganic phosphate significant (r = + 0.53 ± 0.27) nor was it significantly different from the correlation for the water control (r = + 0.14 ± 0.20).

TABLE III

CORRELATIONS * BETWEEN OXYGEN UPTAKE AND THE COLD-TCA-INSOLUBLE PHOSPHATE OF MOORE BARLEY ROOTS TREATED WITH WATER, KBr, DNP, AND KBr + DNP (FIG 7)

MEASUREMENT	TREATMENTS			
	H_2O	0.05 M KBr	DNP **	KBr + DNP
Coeff of correlation, r † ...	0.91 ± 0.04 ††	0.84 ± 0.11	0.94 ± 0.04	0.92 ± 0.07
Coeff of regression, b,[a] μl O_2/micromoles PO_4 × hr	32.6 ± 3.0 ††	16.1 ± 3.4 ‡	54.8 ± 6.4 ‡, ‡‡	64.8 ± 10.5 ‡, ‡‡
Standard error of estimate, μl O_2/hr × 150 seg	8.5	6.8	10.2	11.2
Standard error of estimate, micromoles PO_4/150 seg ..	0.24	0.37	0.18	0.16
Number of cases	27	9	9	8

* All values corrected for number of cases.
** 10^{-5} M DNP treatment for the 0–10 mm region and 5×10^{-6} M for other regions.
† All values significant at P = 0.01.
†† Standard error.
‡ Significantly different from the H_2O treatment at P = 0.01.
‡‡ Significantly different from the KBr treatment at P = 0.01.
[a] Calculated for the line of regression μl O_2 uptake/hr × 150 seg = a + b × (micromoles PO_4/150 seg).



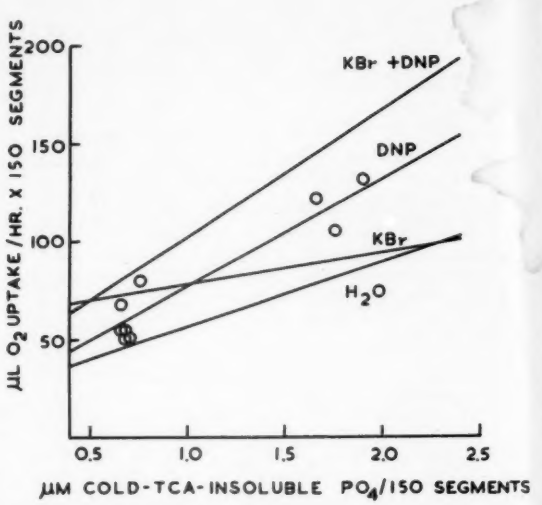


FIG. 6 (*left*). Oxygen uptake by Moore roots in water as a function of the cold-TCA-insoluble phosphate content (scatter points and least squares line for segments from the 0 to 10, 10 to 20, and 20 to 30 mm regions). Details as in table III.

FIG. 7 (*right*). Oxygen uptake by Moore roots treated with water, KBr, DNP, and KBr + DNP as a function of the cold-TCA-insoluble phosphate content (scatter points for the DNP treatment; least squares lines for all treatments and for segments from the 0 to 10, 10 to 20, and 20 to 30 mm regions). Details as in table III.

Respiration of Roots in KBr + DNP Solution: Figure 8 shows that the KBr + DNP treatment incorporated characteristics of the separate KBr and DNP treatments. However, the effects were not additive. Simultaneous application of KBr and DNP resulted in a positive correlation of respiration not only with cold-TCA-insoluble phosphate (table III) but also organic phosphate ($r = +0.77 \pm 0.07$, $P < 0.05$).

DISCUSSION

PHOSPHATE CONTENTS: The phosphate contents of the barley roots were of the same magnitude, 15 to 40 micromoles per gram fresh weight, as those in wheat roots (9). The barley root phosphate (averaged from all root regions) consisted of 24 % TCA-insoluble phosphate and 76 % TCA-soluble phosphate of which 29 % was inorganic phosphate and 71 % was organic phosphate.

The wheat roots of Butler (9) grown in balanced nutrient solution contained essentially the same organic phosphate content as the barley although having up to twice the total phosphate of the barley roots, owing mainly to inorganic phosphate. An important experimental difference was the presence of orthophosphate in the solution bathing Butler's roots whereas the barley roots were initially in phosphate-free solutions. The roots prepared by the methods of Machlis (24) possessed a few root hairs in contrast to the wheat roots. These factors possibly affecting the uptake of inorganic phosphate may partially account for the smaller proportion of inorganic phosphate in the barley roots compared with wheat. Growing barley in phosphate solutions for 5 days increased the inorganic phosphate content without affecting the phosphate ester content (2).

RESPIRATION AND PHOSPHATE CONTENTS: The high positive correlations between respiration rate and cold-TCA-insoluble phosphate per segments was probably a reflection of the association of phosphates with metabolically active materials, e.g., nucleoproteins and lipoproteins. The correlation is probably similar to the association usually found between protein nitrogen and respiration.

The levels of TCA-insoluble phosphorus were of the same magnitude, 0.11 mg/gm fresh wt for the 10 to 20 mm root regions, compared to the DNAP plus RNAP of *Vicia faba* roots, 0.13 mg/gm fresh wt for the 12 to 20 mm root regions (12). These regions in *Vicia faba* and barley are presumably in the fully elongated cell region and thus presumably comparable with respect to physiological age. The relative proportions of RNAP to DNAP were roughly constant (4 : 1) in the various root regions of *Vicia faba* (12). By analogy in the barley the correlation between respiration rate and TCA-insoluble phosphate may not be influenced by phosphorus in the nuclei.

The data of James and Arney (15) from seedlings did not show a correlation between respiration and "residual phosphate" (TCA-insoluble phosphate). However, they found a positive correlation between "esterified phosphate" and carbon dioxide production. It should be noted that James and Arney in their calculations used total seedling respiratory activity over the 12 hours previous to phosphate extractions. The

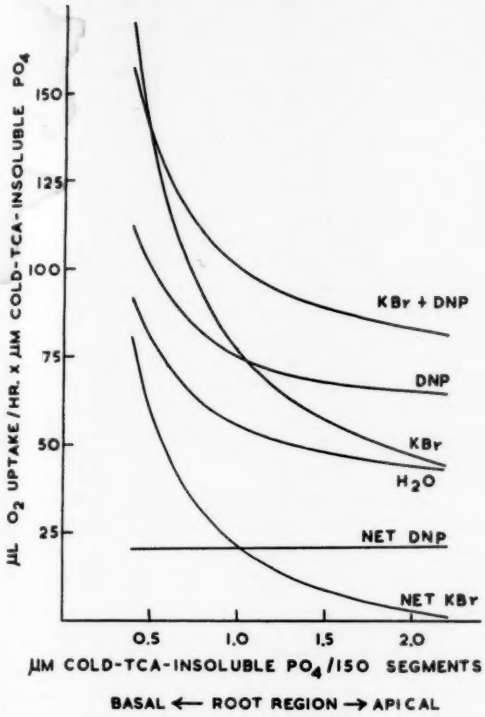


FIG. 8. Specific oxygen uptake by Moore roots treated with water, KBr, DNP, and KBr + DNP as a function of cold-TCA-insoluble phosphate content (transformation of fig 7).

data presented here were from roots only, and the respiratory activity was for the 15 minutes prior to phosphate extraction.

Since there was a high positive correlation between respiration and TCA-insoluble phosphate under all conditions tested, the insoluble phosphate was used to express specific respiratory activity, i.e., respiration rate per respiratory center.

The respiration of roots in water was not found to be correlated with inorganic or organic phosphate. The possibility is not excluded that the respiration rate could be highly correlated with individual phosphate compounds such as ATP. At the time of this investigation, methods were not available to estimate the amounts of ATP in the 200 mg fresh wt, of root tissue used.

EFFECTS OF DNP: If respiration were limited by amounts of acceptor, a negative correlation with organic phosphate and a positive correlation with inorganic phosphate might be established. In the presence of DNP a conversion of ATP to ADP usually occurs, effectively increasing the acceptor and inorganic phosphate content while lowering the total organic phosphate; the respiration might then be expected to become greater as the effective organic phosphate content became lower. Some evidence for a negative correlation of respiration rate with organic phosphate was found in DNP-treated roots. Since the bulk of the organic phosphate was probably not acting as acceptor a higher correlation was probably obscured.

One DNP effect in increasing respiration may result from rendering the mitochondria "leaky" (42). The salt transport system may be intact so that salt lost from the mitochondria activates salt respiration. Thus, an increased respiration in KBr + DNP above that of roots in KBr or DNP alone could result from an increased rate of salt leakage. Alternatively, hydrogen ions produced in salt respiration may suppress the ionization of weak electrolytes so that DNP has more ready access to sites of action (cf. synergistic effects of DNP + KBr, fig 8, and Robertson, Wilkins and Weeks (37)).

CONTROL OF BARLEY ROOT RESPIRATION: In contrast to the oxygen uptake rates per segments, in water, the specific respiratory activities (i.e., activity/TCA-insoluble phosphate) were greater for the root regions located progressively more distant from the root apex. The increase in specific respiratory activity was pronounced compared with the gradient of pea root respiration per protein nitrogen (7). Cytoplasmic protein not concerned directly with respiration may have diluted any gradient for the pea.

If phosphorylative coupling reactions were the limiting control on respiration of the barley roots, the specific respiratory rate (not the rate per segments) induced by DNP should have reached the same maximum everywhere along the root axis. Since the marked differential along the roots did not disappear under DNP treatment, control by phosphorylative coupling reactions probably was not dominating and another control system exists. The lack of correlation between the respiration rate of roots in water and the content of inorganic and organic phosphate is consistent with this view.

Upon DNP treatment respiration was increased and became partially correlated with organic phosphate. Thus, although not quantitatively assessable from the data, the respiration of the roots showed the properties expected if it were partially controlled by phosphorylative coupling reactions. It is suggested that the degree of respiratory control by phosphorylative coupling reactions cannot be determined by only the DNP effect on respiratory rate.

SALT RESPIRATION: The net salt respiration of barley root segments was minimal for the apical regions although the endogenous respiration was maximal compared with the more basal regions. As in other tissues only a slight amount of salt (2×10^{-3} M KBr) was required to induce a near-maximal salt respiration.

The differential increase in respiration along the root axis induced by KBr can be explained if salt accumulation were a function of vacuolation as proposed by Brown and Cartwright (8). The apparent lack of salt respiration in the non-vacuolated regions can be explained if the salt transporting system and

salt respiration were already saturated. Possibly the mitochondria, in the cells with little or no vacuolation, have a salt respiration maintaining a concentration of salt in the mitochondria (36). If transfer of salt to vacuoles cannot occur (32), salt respiration need only balance a salt leakage from the mitochondria (36) and could be the portion of the respiration in water inhibited by cyanide (27).

The lack of salt respiration in the apex cannot be ascribed to the apex already having a maximal respiration rate since DNP increased the respiration of the apex and the specific respiratory activity of the apex was low in comparison with other regions.

Respiration to offset leakage from vacuoles may be slight since leakage across the tonoplast membrane appears to be slow (1, 41). It is pertinent that some vacuolated tissues possess a basal respiration in water which is insensitive to cyanide (34), i.e., a cyanide-sensitive salt respiration may become nil after salt has been transferred to the vacuoles (33).

The effects of KBr and DNP upon the respiratory activity cannot be due entirely to analogous effects. It is unlikely that salt activates salt respiration by an energy drain whereby ADP is made available since the correlation of root respiration with organic phosphate content for KBr-treated roots was not similar to the correlation for the DNP-treated roots.

Comparative studies indicate that spatial separation of hydrogen ions from electrons during their passage from substrates to oxygen in the cytochrome system may be required for salt accumulation in plants or acid secretion in gastric mucosae of animals (32). The effect of DNP, apart from preventing the normal production of ATP, is to suppress salt accumulation (37). Thus DNP may abolish accumulation by preventing the prolonged separation of hydrogen ions from electrons during salt respiration rather than by an inhibition of net ATP production. Keynes (18) maintains that ". . . there is no compelling proof that ATP plays a direct role in driving active transport systems. . . ."

Cytochrome System and Salt Respiration: This investigation arose from the identification of salt respiration with cytochrome respiration. If salt respiration is cytochrome respiration in barley roots in what way does salt increase the activity of the cytochrome system? The evidence presented does not indicate that salt respiration is directly controlled by phosphorylative coupling reactions. The following discussion concerns various pertinent evidence on the above points.

Evidence for a Salt-Cytochrome Respiration in Barley: While several different terminal oxidases may function in barley roots (16), cytochrome oxidase is assumed to mediate salt respiration. The isolation of a "mitochondrial" cytochrome oxidase from 2- to 10-day-old barley roots (14) is consistent with a mitochondrial cytochrome system associated with salt accumulation as proposed by Robertson (31). Moreover, although only a fraction of the respiration of roots in salt was dependent upon the cytochrome system as shown by the effects of carbon monoxide, the uptake of potassium and bromide was completely dependent (29).

There is agreement on the presence of cytochrome oxidase in roots of barley up to 6 days old (13, 14, 16, and 29). It is possible that conflicting evidence for older barley is due to varietal differences of the barley examined and the use of different portions of the root. James and Boulter (16) used only the apical meristematic region and found progressively less cytochrome activity with increasing age. Continual cell division may deplete the cytochrome complement faster than it is replaced so that the root tips of progressively older seedlings display less and less cytochrome activity (Spiegelman, 40).

Hypothetically, the activity of any metallo-terminal oxidase system might be facilitated by salt as Lundegårdh (22) suggested for the cytochrome system. Consequently it is interesting that ascorbic oxidase was claimed to support salt accumulation in barley roots (39). However, this claim rests upon the assumptions that (a) diethyldithiocarbamate inhibited respiration and salt accumulation as it inhibited ascorbic oxidase, (b) cytochrome oxidase was absent, and (c) the uptake of salt was by active salt accumulation.

The specificity of diethyldithiocarbamate in vivo for ascorbic oxidase cannot yet be assumed. Not only may it inhibit succinic dehydrogenase after being oxidized (17), but it may also inhibit copper enzymes, other than ascorbic oxidase, which operate a sequence of electron transfers to a cytochrome system (the constitution of DPNH oxidase (11)).

Russell (39) may be correct in that ascorbic oxidase supports *salt uptake* but the root tip which he studied is different from the more mature root regions not only in its salt respiration being slight and its enzyme complement possibly being different (16), but probably also in its salt accumulation being slight owing to lack of vacuolar development (8). The difference thus must be clearly distinguished between *salt uptake*, which may occur by *exchange adsorption* and a *Donnan adjustment*, and *active accumulation* of salts free in solution against concentration and chemical gradients (Robertson, 32). Russell's proposals based upon excised barley root tip experiments are not necessarily applicable to the mechanism of salt accumulation but perhaps to other metabolic effects, for example, production of binding sites in the cytoplasm.

Mechanism of Salt Increasing Respiration: The effect of salt may be direct or indirect in increasing respiration associated with salt accumulation. Suggestions for a direct effect are here based ultimately upon the 4 : 1 ratio of accumulation to salt respiration rates demonstrated by Robertson and Wilkins (35). The stoichiometry would be difficult to explain by an indirect effect such as a Helmholtz double-ion-layer phenomenon whereby salts modify the zeta potential of interfaces between the cytoplasm and, for example, mitochondria. If, however, the 4 : 1 ratio

were fortuitous then effects of ionic milieu must be considered. If the stoichiometry of accumulation to oxygen uptake is correct then a direct interaction must be involved in accumulation and possibly also in the stimulation of respiration. For example, salt may act through some variation of ion-pair formation.

In the case of a specific salt interaction, a profitable approach appears to be through study of the cytochromes. Some evidence has already been found for salt-cytochrome interactions. During oxidation in the cytochrome system a complex with cytochrome may incorporate inorganic anions and cations and may facilitate oxidation (6). The way in which a chloride complex facilitates the oxidation of a ferrous ion by a ferric ion (43) may be analogous to oxidations of the cytochromes in salt respiration. Further evidence for inorganic ion interactions with the cytochrome system are anionic effects on the magnetic susceptibility and Soret band of ferricytochrome c (4), stimulating effects of cations upon the mitochondrial cytochrome system (30) and of anions upon the autoxidation of cytochrome c (5), salt stimulations of the mitochondrial cyochrome system of pea seedlings (10).

Summary

Phosphate contents of barley roots varied according to the region, ranging between 15 to 40 micromoles/gm fresh weight. The percentage distribution of the different phosphate fractions (averaged over the 0 to 30 mm root region) was 24 % cold TCA-insoluble phosphate and 76 % TCA-soluble phosphate of which 29 % was inorganic and 71 % organic phosphate.

A low concentration of KBr (ca 2×10^{-3} M) induced a near-maximal salt respiration and DNP about 10^{-5} M induced maximal increases in respiration depending upon the root region.

Respiration under all conditions tested was significantly correlated ($r = +0.84 \pm 0.11$ to $r = +0.94 \pm 0.04$) with the TCA-insoluble phosphate content, presumably reflecting the association between respiration and the metabolizing materials, e.g., nucleoproteins and lipoproteins. Respiration was generally not correlated with inorganic or organic phosphate content.

Barley roots not only displayed a respiratory gradient per segment which decreased progressively from the apex but also a salt respiration which increased progressively from the apex. Estimation of the activity per respiratory center, however, showed that the apex had the least specific respiratory activity under all conditions tested.

The effects of DNP indicated a minor degree of control of respiration by phosphorylative coupling reactions. Since the specific respiratory gradient along the root axis was not abolished by DNP treatment, the presence of another control system is inferred.

Lack of salt respiration in the root apex cannot be attributed to respiration already occurring maximally since DNP increased respiration and the specific respiratory activity was low compared to other root regions.

The effects of KBr and DNP are discussed in relation to the nature of salt respiration.

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Literature Cited

1. Arisz, W. H. Transport of chloride in the 'symplasm' of Vallisneria leaves. Nature 174: 223–224. 1954.
2. Arney, S. E. Phosphate fractions in barley. Biochem. Jour. 33: 1078–1086. 1939.
3. Beevers, H. 2,4-Dinitrophenol and plant respiration. Amer. Jour. Bot. 40: 91–96. 1953.
4. Boeri, E., Ehrenberg, A., Paul, K. G., and Theorell, H. On the compounds of ferricytochrome c appearing in acid solution. Biochim. Biophys. Acta 12: 273–282. 1953.
5. Boeri, E. and Tosi, L. The effect of salts on the autoxidation of cytochrome c. Arch. Biochem. Biophys. 52: 83–92. 1954.
6. Boyer, P. D., Falcone, A. B., and Harrison, W. H. Reversal and mechanism of oxidative phosphorylation. Nature 174: 401–402. 1954.
7. Brown, R. and Broadbent, D. The development of cells in the growing zones of the root. Jour. Exptl. Bot. 1: 249–263. 1950.
8. Brown, R. and Cartwright, P. M. The absorption of potassium by cells in the apex of the root. Jour. Exptl. Bot. 4: 197–221. 1953.
9. Butler, G. W. Ion uptake by young wheat roots. III. Phosphate absorption by excised roots. Physiol. Plantarum 6: 637–661. 1953.
10. Butler, G. W. The connexion between respiration and salt accumulation. I. Preliminary note. Physiol. Plantarum 6: 662–671. 1953.
11. Green, D. E., Mackler, B., Repaske, R., and Mahler, H. R. DPNH oxidase. Biochim. Biophys. Acta 15: 435–437. 1954.
12. Holmes, B. E., Mee, L. K., Hornsey, S., and Gray, L. H. The nucleic acid content of cells in the meristematic elongating and fully elongated segments of roots of *Vicia faba*. Exptl. Cell Research 8: 101–113. 1955.
13. Honda, S. I. Ascorbic acid oxidase in barley roots. Plant Physiol. 30: 174–181. 1955.
14. Honda, S. I. Succinoxidase and cytochrome oxidase in barley roots. Plant Physiol. 30: 402–410. 1955.
15. James, W. O. and Arney, S. E. Phosphorylation

and respiration in barley. New Phytologist 38: 340–351. 1939.

16. James, W. O. and Boulter, D. Further studies of the terminal oxidases in the embryos and young roots of barley. New Phytologist 54: 1–12. 1955.
17. Keilin, D. and Hartree, E. F. Succinic dehydrogenase–cytochrome system of cells. Intracellular respiratory system catalysing aerobic oxidation of succinic acid. Proc. Roy. Soc. (London) B 129: 277–306. 1940.
18. Keynes, R. D. Ionic permeability of nerve membranes. In: Recent Developments in Cell Physiology, J. A. Kitching, ed. P. 47. Butterworths Scientific Publications, London. 1954.
19. Lardy, H. A. and Wellman, H. Oxidative phosphorylations: Role of inorganic phosphate and acceptor systems in control of metabolic rates. Jour. Biol. Chem. 195: 215–224. 1952.
20. Lehninger, A. L., Hassen, M. ul, and Suddarth, H. C. Phosphorylation coupled to the oxidation of ascorbic acid by isolated mitochondria. Jour. Biol. Chem. 210: 911–922. 1954.
21. Lowry, O. H. and Lopez, J. A. The determination of inorganic phosphate in the presence of labile phosphate esters. Jour. Biol. Chem. 162: 421–428. 1946.
22. Lundegårdh, H. Absorption, transport, and exudation of inorganic ions by the roots. Arkiv Bot. 32: No. 12. 1945.
23. Lundegårdh, H. and Burström, H. Untersuchungen über die Salzaufnahme der Pflanzen. III. Mitteilung: Quantitative Beziehungen zwischen Atmung und Anionen-Aufnahme. Biochem. Zeits. 261: 235–251. 1933.
24. Machlis, L. The influence of some respiratory inhibitors and intermediates on respiration and salt accumulation of excised barley roots. Amer. Jour. Bot. 31: 183–192. 1944.
25. Machlis, L. The respiratory gradient in barley roots. Amer. Jour. Bot. 31: 281–282. 1944.
26. Maley, G. F. and Lardy, H. Phosphorylation coupled with the oxidation of reduced cytochrome c. Jour. Biol. Chem. 210: 903–909. 1954.
27. Milthorpe, J. and Robertson, R. N. Studies in the metabolism of plant cells, 6. Salt respiration and accumulation in barley roots. Australian Jour. Exptl. Biol. Med. Sci. 26: 189–197. 1948.
28. Newcomb, E. H. Tobacco callus respiration and its response to 2,4-dinitrophenol. Amer. Jour. Bot. 37: 264–271. 1950.
29. Ordin, L. and Jacobson, L. Inhibition of ion absorption and respiration in barley roots. Plant Physiol. 30: 21–27. 1955.
30. Pressman, B. C. and Lardy, H. A. Influence of potassium and other alkali cations on respiration of mitochondria. Jour. Biol. Chem. 197: 547–556. 1952.
31. Robertson, R. N. Mechanism of absorption and transport of inorganic nutrients in plants. Ann. Rev. Plant Physiol. 2: 1–24. 1951.
32. Robertson, R. N. The mechanism of absorption. In: Handbuch der Pflanzenphysiologie, E. Ruhland, ed.; W. J. Bogen, sub. ed. Vol. 2, Part III. H. Springer Verlag, Heidelberg. (In press.) 1955.
33. Robertson, R. N. and Thorn, M. Studies in the metabolism of plant cells, 4. The reversibility of the salt respiration. Australian Jour. Exptl. Biol. Med. Sci. 23: 305–309. 1945.
34. Robertson, R. N. and Turner, J. S. Studies in the metabolism of plant cells, 3. The effects of cyanide on the accumulation of potassium chloride and on respiration; the nature of the salt respiration. Australian Jour. Exptl. Biol. Med. Sci. 23: 63–73. 1945.
35. Robertson, R. N. and Wilkins, M. J. Studies in the metabolism of plant cells, 7. The quantitative relation between salt accumulation and salt respiration. Australian Jour. Sci. Research B 1: 17–37. 1948.
36. Robertson, R. N., Wilkins, M. J., Hope, A. B., and Nestel, L. Studies in the metabolism of plant cells, 10. Respiratory activity and ionic relations of plant mitochondria. Australian Jour. Biol. Sci. 8: 164–185. 1955.
37. Robertson, R. N., Wilkins, M. J., and Weeks, D. C. Studies in the metabolism of plant cells, 9. The effects of 2,4-dinitrophenol on salt accumulation and salt respiration. Australian Jour. Sci. Research B 4: 248–264. 1951.
38. Rowan, K. S. and Seaman, D. E. The relationship between phosphorylation and the Pasteur effect in pea seed. Pp. 49–50. Abstr. 31st Meetings Australian and New Zealand Assoc. Adv. Science, Section N, Physiology and Biochemistry. 1955.
39. Russell, R. S. The relationship between metabolism and the accumulation of ions by plants. In: Active Transport and Secretion, Symposia Soc. Exptl. Biol. 8: 343–366. 1954.
40. Spiegelman, S. Modern aspects of enzymatic adaptation. In: The Enzymes, J. B. Sumner and K. Myrback, eds. Vol. I. Part 1, Pp. 298–299. Academic Press Inc., New York. 1950.
41. Sutcliffe, J. F. The exchangeability of potassium and bromide ions in cells of red beetroot tissue. Jour. Exptl. Bot. 5: 313–326. 1954.
42. Tepley, L. J. Studies on the cyclophorase system. XIV. Mechanism of the action of 2,4-dinitrophenol. Arch. Biochem. 24: 383–388. 1949.
43. Zwolinski, B J., Marcus, R. J., and Eyring, H. Inorganic oxidation-reduction reactions in solution. Electron transfers. Chem. Rev. 55: 157–180. 1955.

QUANTITATIVE DETERMINATION OF BUCKWHEAT FLAVONOID SUBSTANCES BY DIRECT ABSORPTIOMETRY OF PAPER CHROMATOGRAMS[1,2]

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Analytical methods developed for flavonoid compounds have in general not been adequate for complex mixtures of these substances. A gravimetric procedure for rutin in plant materials (12) is time-consuming and unsatisfactory when the rutin concentration is small. Various non-specific methods (5, 20) and spectrophotometric techniques (18, 19) are applicable only to substances occurring singly or at most in pairs. Since the number of such described methods is large, only representative examples are cited here.

For work with buckwheat seedling material in which five flavonoids occur together (17) none of the above procedures could be applied directly. Attempts at preliminary separation by solvent extraction or various other means failed completely; only by paper-chromatographic techniques could the five substances be separated, and then only in small amounts. Elution procedures involving leaching of separated chromatogram strips with ethanol (13), ethanol acidified with HCl (7), aluminum chloride solution (8), or ethanol-ethyl acetate (6), could not be used since none of these removed all of the materials from the paper quantitatively.

It was necessary, therefore, to search for a method of analysis which does not require removal of the substances from the developed chromatograms. Many such methods have been described, all of which utilize a relation between amount of substance and some measure of zone dimension. Several devices have been used to measure the light absorption of substances on filter paper strips (3, 4, 14, 15, 16). In the present work modifications of two such techniques—those of Tennent, Whitla, and Florey (16) and of Paladini and Leloir (14)—have been applied to the estimation of rutin and four other buckwheat flavonoids. This direct absorptiometric procedure has proved adequate for the simultaneous determination of small quantities of these five substances in mixtures.

EXPERIMENTAL

GENERAL DESCRIPTION OF PROCEDURE: For scanning the paper strips an adapter to fit the 1-cm cell compartment of the Beckman DU spectrophotometer is used. This is essentially similar to that described by Tennent, Whitla, and Florey (16), except that the window is larger (0.9 cm wide by 2.1 cm high) in order to allow use of wider paper strips (14). Known volumes of the solutions to be analyzed are spotted on strips of Whatman No. 1 filter paper 2.5 cm wide and of suitable length; these are subjected to chromatography and dried. A blank strip is carried through the chromatographic procedure. For scanning, the spectrophotometer is set at zero at an appropriate wavelength against a piece of unused filter paper, with the selector switch at 1.0 if possible. A strip-chromatogram is then wound onto the spools of the adapter and oriented with the starting line at the edge of the window, the latter being placed on the side of the cell compartment nearer the phototube housing. The shutter is opened and the instrument brought to balance with the density knob. The strip is then advanced an arbitrary distance as measured by the revolution-counter of the adapter. By repetition of this procedure the absorbance values at equal intervals along the length of the strip are recorded. The instrument is reset at zero before each scanning, and the blank chromatogram is treated in the same manner as the others. Inspection of the absorbance values will reveal the limits of each zone on a particular strip; these locations may be checked by treating the scanned strips with a suitable reagent. For quantitative estimation the areas under the curve absorbance vs. distance along the chromatogram are measured; these raw areas are corrected by subtracting the area values for similar portions of the blank strip. Such use of a blank makes the filter paper used in balancing the spectrophotometer a less critical factor. Finally, quantities corresponding to the measured areas are calculated by use of a standard curve for each substance.

In the present work the upper layer of the system chloroform–isobutanol–water (2–4–4 by volume) was used for chromatography. The prepared strips, 57 cm long, were equilibrated with the solvent vapors for 24 hours; development was downward for 15 to 18 hours, the solvent being allowed to drip from the bottoms of the strips. Scanning was carried out at 350 mμ, using the tungsten lamp; absorbance values were read at equilinear intervals of approximately 5 mm. Locations of the zones were checked through use of aluminum chloride reagent and observation under ultraviolet light. Since absorbance readings were made at equal intervals of distance, it was convenient to estimate areas under the absorbance vs. distance curve by the purely computational trapezoidal approximation (21). Any of several other methods for measuring areas could, of course, be employed if desired.

STANDARD CURVE FOR RUTIN: Five-microliter volumes of five rutin solutions of known concentration were applied to paper strips which, along with a

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blank, were carried through the above chromatographic and scanning procedure. Eight replications were prepared in all, each on a different day. Different batches of solvent were used for each set, and such routine matters as order of spotting on strips, position in the chromatographic chamber, and order of scanning were arranged by reference to a table of random numbers.

Application to Buckwheat Extract: In order to test the suitability of the method for use with plant material, an ethanolic extract of cotyledons of green buckwheat seedlings (*Fagopyrum esculentum* Moench var. Japanese), known to contain rutin and four other flavonoids (17), was prepared. Four different volumes of this extract were analyzed in three replications with procedures randomized.

Recovery of Rutin: Five amounts of pure rutin were measured, each in three replications with randomized procedures. Rutin recovered was calculated from the standardization data.

Recovery of Rutin in Buckwheat Extract: Three known amounts of pure rutin were applied to strips, in each case with a specific volume of the extract of green buckwheat cotyledons. Three replications were analyzed, following randomized procedures, and the amounts of rutin recovered calculated from the standardization data. In analyzing the results of this experiment the figures for total actual rutin were obtained by summing the values for rutin already present in the volume of extract used and the values for rutin added.

Results and Discussion

The principal justification of the proposed method is empirical. Nevertheless, a brief statement of certain relevant facts may indicate some of the possible sources of error in the described procedure: (a) the physical condition of a substance in a spot on a chromatogram is unknown; (b) distribution of material in a chromatogram spot is irregular; (c) variations in background absorption occur, owing to nonuniformity of structure of the filter paper; (d) the spectrophotometer may be subject to zero drift; (e) the light beam of the spectrophotometer possesses finite dimensions; (f) in certain positions only part of the spot width may be covered by the light beam; (g) in other positions, especially at the leading and trailing edges, not all of the beam passes through the spot. The bearing of these and other considerations on error in methods of this sort has been discussed adequately by others (1, 2, 3, 4, 14, 16). It is interesting to note that Liebhafsky and Pfeiffer (10) have suggested a theoretical foundation for such techniques as this.

In the case of the buckwheat flavonoids a single scanning of each chromatogram strip at 350 mμ is adequate for all zones even though maximum absorption does not occur at this wavelength in all instances. Absorbance vs. distance curves for two amounts of pure rutin and for a buckwheat extract are shown in figure 1 A and 1 C. The peaks corresponding to the five flavonoid substances are easily distinguished, and the effect of quantity on the shape and height of the rutin curves is apparent. In the latter connection it should be noted that in the solvent system used flavonoid substances tend to "streak" into elongated zones when chromatographed singly. In mixtures, however, such streaking is reduced and the individual spots are distinctly separated provided too large a quantity of material is not used. Compare, for example, the elongated rutin zone obtained when that substance is chromatographed alone (fig 1 A) with the short, distinct zone given by rutin in a buckwheat extract (fig 1 C, zone e). In the present work any chromatogram which showed overlapping of spots was discarded.

Figure 1 B depicts curves obtained by scanning

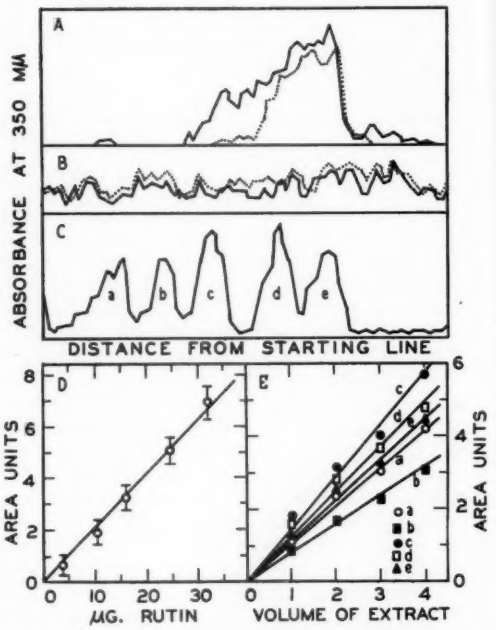

Fig. 1. A–C. Absorbance vs distance plots for paper chromatograms developed with the upper layer of chloroform–isobutanol–water (2–4–4 by volume), then scanned at 350 mμ. A. Two amounts of pure rutin: solid line, 16 μg; broken line, 10 μg. "Streaking" is quite noticeable in the case of the larger quantity. B. Blank paper strip: solid line, before development with solvent; broken line, after development. C. Extract of green buckwheat cotyledons showing peaks corresponding to the five flavonoid substances. "Streaking" of spots is at a minimum.

D. Rutin standardization data. Each point is the mean of 8 observations; vertical bars represent 95% confidence limits for the means. The fitted least-squares line through the origin is also shown.

E. Area-quantity plots for the 5 flavonoid substances present in the extract of green buckwheat cotyledons. Each point is the mean of triplicate analyses. For each substance the fitted line through the origin is shown.

the same blank paper strips before and after development with the chloroform–isobutanol–water system; no effect of this solvent on absorption by the paper at the stated wavelength is noted. The obvious variation in absorption at different points on the strip should probably be attributed to non-uniform paper structure.

Data for the rutin standardization analyses are presented in figure 1 D. Variation among the replicates for each concentration was rather large, but a plot of mean areal units vs. micrograms rutin approximated a linear relationship. A straight line of the form $y = bx$ was therefore fitted to the data by the method of least squares. In order to test the hypothesis that the mean areas fall on a line through the origin analysis-of-variance techniques were employed; at the 5 % level the hypothesis that the regression is linear and through the origin was accepted. Subsequent calculations for rutin were made on the basis of the fitted equation.

Results of the test of the applicability of the method to buckwheat extracts are depicted in figure 1 E; here are plotted the means of the triplicate analyses for each of the five flavonoid substances present in the extract, together with the fitted least-squares lines through the origin. For each material deviations from a regression of the form $y = bx$ were adjudged to be insignificant at the 5 % level after analysis-of-variance tests. These results suggest that the method may be applied to a mixture to secure quantitative values of a relative nature even when known amounts of some or all of the components cannot be obtained and standard curves are therefore unavailable.

Data obtained from the recovery trials are presented in table I. In all cases absolute errors were never greater than about 3 μgm. Ratios of the amount of rutin found to the amount actually applied varied from a low of 0.80 to a high of 1.10 (80 to 110 % recovery).

Youden (22) and others (11) have pointed out that statistical techniques of regression analysis may afford a systematic way of passing judgment on the suitability of a proposed analytical method. Lark (9) has added some warnings concerning such an application and has amplified the evaluation scheme. In the present study the procedures of the latter author were followed; since details and a theoretical discussion are contained in Lark's paper, only a brief description is presented below.

TABLE I

RECOVERY OF RUTIN FROM CHROMATOGRAMS OF PURE RUTIN AND FROM CHROMATOGRAMS OF BUCKWHEAT EXTRACT TO WHICH EXTRA AMOUNTS OF PURE RUTIN WERE ADDED

ACTUAL RUTIN μGM	RUTIN FOUND μGM	RATIO, ACTUAL/FOUND	MEAN ERROR, FOUND – ACTUAL μGM
Chromatograms of pure rutin			
3	3	1.00	0.3
10	8	0.80	1.7
16	16	1.00	0.3
25	24	0.96	1.3
31	34	1.10	2.7
Buckwheat extract + rutin			
9	10	0.90	1.3
16	16	1.00	0.0
22	19	0.86	3.3

1. The hypothesis was proposed that the regression of the amount of rutin found (y) on the actual amount (x) is linear in the range of quantities employed. With the usual least-squares assumptions, therefore, lines of the form $y = a + bx$ were fitted to the recovery data for pure rutin and for rutin added to the buckwheat extract (table I). Variance unexplained by the regression was compared with variance among replicates. In both cases the hypothesis of linearity was accepted at the 5 % level.

2. The hypothesis was proposed that the true values of the regression coefficients (b) of the above equations are equal to one. If the analytical technique is free of systematic error, such a condition should exist, although the reverse is not necessarily true (9). On the basis of t-tests this hypothesis was accepted at the 5 % level in both cases.

3. Lark (9) has shown that it is instructive to consider the regression of total error of analytical determination ($z = y - x$) on the actual amount; the total error, z, includes systematic error and random error. One hypothesis which might be assumed is that systematic error is linearly related to the actual amount. In the present investigation this possibility was studied by fitting equations of the form $z = a + b'x$ to the rutin recovery data. If the method were free of relative-type systematic error which varies with the amount of material taken for analysis, the regression coefficients, b′, of these equations would be equal to zero. The hypothesis that they are indeed zero was evaluated by means of t-tests and accepted at the 5 % level in both cases.

4. Alternatively, the hypothesis may be adopted that the systematic error is constant ($z = a'$). In this event the best estimate of the error is the mean of the z values (9). Such mean errors were calculated for the rutin recovery data, and the hypothesis that they are equal to zero was tested through use of the statistic t. For both recovery experiments this hypothesis was accepted at the 5 % level.

5. A good idea of the precision of a method may be obtained by inverting the regression equation of y on x and calculating confidence intervals for predictions of actual amounts, x, in new samples (9). Results of such an analysis of the data for pure rutin are presented in table II. The data of the table were calculated under the assumption that the values for "rutin found" would be means of triplicate analyses.

In summary, it has been concluded that in the range 3 to 30 μgm the suggested analytical procedure yields a recovery relationship which is linear with a slope equal to one, and that the method is free of both relative and constant types of systematic error. It must be remembered that the basis for these decisions is the error among replicate determinations. Thus,

TABLE II

PREDICTION OF TRUE RUTIN VALUES IN NEW SAMPLES FROM THOSE WHICH MIGHT BE FOUND BY ANALYSIS OF CHROMATOGRAMS OF PURE RUTIN

RUTIN FOUND	TRUE RUTIN, BEST ESTIMATE	90 % CONFIDENCE LIMITS
μgm	*μgm*	*μgm*
5	5.9	1.8– 9.6
10	10.5	6.2–14.7
15	15.2	11.5–28.9
20	19.9	16.2–23.7
25	24.6	20.9–28.5
30	29.2	25.5–33.4

systematic error may actually be present; if so, it is insignificant in comparison with the variation among replicates.

Obviously the precision of determinations made with this procedure is not as good as might be desired. Indeed, the confidence intervals of table II are broad enough that the method should be regarded as semiquantitative in nature and not employed if a more exact procedure can be applied. However, the principal feature which recommends the method may be a compelling one: since it can be used for any complex mixture of flavonoids capable of resolution by the paper-chromatographic technique, it may—as in the case of buckwheat seedling extracts—succeed when other procedures fail. If the choice is to analyze or not to analyze, one cannot escape the conclusion that inexact knowledge is a somewhat happier possession than complete ignorance.

SUMMARY

The direct absorptiometry of paper chromatograms has been applied to the quantitative analysis of flavonoid compounds. The method has proved satisfactory for rutin alone and in buckwheat extracts. It can also be used for relative measurements on four other flavonoid substances present in buckwheat seedlings and is probably applicable to plant materials in general. Because of the degree of precision obtained, the method should be regarded as semi-quantitative in nature.

Dr. Joseph Naghski, United States Department of Agriculture, generously provided the sample of pure rutin used.

LITERATURE CITED

1. BRADFIELD, A. E. and FLOOD, A. E. The direct measurement of the absorption spectra of some plant phenols on paper strip chromatograms. Jour. Chem. Soc. (London) 1952: 4740–4744. 1952.
2. BROWN, J. A. and MARSH, M. M. Paper chromatographic separation and determination of some water-soluble vitamins. Anal. Chem. 24: 1952–1956. 1952.
3. BROWN, J. A. and MARSH, M. M. Automatic measurement of light absorption and fluorescence on paper chromatograms. Anal. Chem. 25: 1865–1869. 1953.
4. CAMPBELL, H. and SIMPSON, J. A. Spectrophotometric attachment for use in paper chromatography. Chemistry & Industry 1953: 342–343. 1953.
5. DAVIS, W. B. Determination of flavanones in citrus fruits. Anal. Chem. 19: 476–478. 1947.
6. DILLAHA, J., GAGE, T. B., and WENDER, S. H. Quantitative paper chromatography of certain flavonol aglycons. Proc. Oklahoma Acad. Sci. 32: 102–104. 1951.
7. GAGE, T. B. and WENDER, S. H. Paper chromatography of flavonoid pigments. II. Separation and quantitative estimation of rutin and quercetin. Federation Proc. 8: 293–294. 1949.
8. GAGE, T. B. and WENDER, S. H. Quantitative determination of certain flavonol-3-glycosides by paper partition chromatography. Anal. Chem. 22: 708–711. 1950.
9. LARK, P. D. Application of statistical analysis to analytical data. Anal. Chem. 26: 1712–1715. 1954.
10. LIEBHAFSKY, H. A. and PFEIFFER, H. G. Beer's law in analytical chemistry. Jour. Chem. Educ. 30: 450–452. 1953.
11. LINNIG, F. J., MANDEL, J., and PETERSON, J. M. A plan for studying the accuracy and precision of an analytical procedure. Anal. Chem. 26: 1102–1110. 1954.
12. NAGHSKI, J., FENSKE, C. S., JR., KREWSON, C. F., and COUCH, J. F. Determination of rutin in plant materials. U. S. Dept. Agr., Bur. Agr. and Ind. Chem., Mimeographed Circ. AIC236. 1949.
13. NORDSTRÖM, C. G., and SWAIN, T. The flavonoid glycosides of Dahlia variabilis. I. General introduction. Cyanidin, apigenin, and luteolin glycosides from the variety "Dandy." Jour. Chem. Soc. (London) 1953: 2764–2773. 1953.
14. PALADINI, A. C. and LELOIR, L. F. Detection of ultra-violet absorbing substances on paper chromatograms. Anal. Chem. 24: 1024–1025. 1952.
15. PARKE, T. U. and DAVIS, W. W. Automatic spectrophotometry of paper strip chromatograms. Anal. Chem. 24: 2019. 1952.
16. TENNENT, D. M., WHITLA, J. B., and FLOREY, K. Two techniques in paper chromatography. Application to ketosteroids. Anal. Chem. 23: 1748–1749. 1951.
17. TROYER, J. R. The distribution of rutin and other flavonoid substances in buckwheat seedlings. Plant Physiol. 30: 168–173. 1955.
18. TURNER, A., JR. Determination of rutin in buckwheat leaf meal and other plant materials. Anal. Chem. 24: 1444–1445. 1952.
19. TURNER, A., JR. Report on rutin in tablets. Jour. Assoc. Off. Agr. Chem. 36: 699–707. 1953.
20. WILSON, C. W., WEATHERBY, L. S., and BOCK, W. Z. Determination of certain quercetin-like substances using a Klett-Summerson photoelectric colorimeter. Ind. Eng. Chem., Anal. Ed. 14: 425–426. 1942.
21. WORTHING, A. G. and GEFFNER, J. Treatment of Experimental Data. Pp. 1–342. John Wiley and Sons, New York. 1943.
22. YOUDEN, W. J. Technique for testing the accuracy of experimental data. Anal. Chem. 19: 946–950. 1947.

QUANTITATIVE CHANGES IN BUCKWHEAT FLAVONOID SUBSTANCES DURING SEEDLING DEVELOPMENT[1,2]

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Relatively few studies have been made of changes in the flavonoid constitution of plants as they develop. Bate-Smith (2) has presented a qualitative picture of such changes in the maturing flower of Coreopsis. Hesperidin (5) and naringin (8) in citrus fruits and rutin in Nicotiana (1) have been examined quantitatively under field conditions.

Since rutin has become of commercial interest, a number of workers have followed the formation of this glycoside in mature buckwheat plants (7, 10, 11, 13). Workers of the United States Department of Agriculture found that in Japanese (3, 12) and Tartary (4, 12) buckwheats most of the rutin occurs in the leaves and blossoms; little was found in the stems. Rutin content increases steadily, reaching a peak as the plant nears maturity and thereafter declining. More detailed studies of the distribution of the glycoside within the mature buckwheat plant have not been reported.

The fact that rutin and four other flavonoids occur in plants of Japanese buckwheat only three days old while the flavonoid content of the ungerminated seed is extremely small (14) suggested that the seedling stage may be a critical one as far as flavonoid constitution is concerned. Accordingly, quantitative levels of the buckwheat substances in the several organs were followed during germination and initial development of green and etiolated seedlings. This investigation revealed a rapid appearance and quantitative increase in all five of the buckwheat flavonoids in plants of both types.

MATERIALS AND METHODS

SEEDLING CULTURE: Seeds of *Fagopyrum esculentum* Moench var. Japanese, purchased from the Peter Henderson, Stumpp and Walter Co., were used; without removal of the pericarps they were soaked for 5 hours in distilled water, spread on moist filter paper, and placed in the dark at 27 ± 2° C. After 2 days lots of the germinated seeds were selected for further use. Etiolated seedlings were cultured in moist sand contained in Pyrex dishes 20 cm square and 5 cm deep; these were kept in the dark at 27 ± 2° C and received only daily portions of distilled water. Green seedlings were cultured in moist sand in large clay pots which were placed in the greenhouse and watered daily with tap water. Positional arrangement in the dark chamber and on the greenhouse bench of lots of seedlings tagged for specific harvest dates was assigned through use of a table of random numbers.

HARVESTING PROCEDURES: For each set of seedlings the plants were carefully removed and the sand washed from the roots. The lengths of the roots and hypocotyls of 50 seedlings were measured at this time. The plants were then grouped into four lots, two of 50 to 100 and two of 200 to 300 individuals. As rapidly as possible all were divided into their constituent organs. As soon as all free water appeared to be gone from the plant surfaces, fresh weights were obtained for all four lots. The two smaller groups were then dried at 68° C for 48 hours and stored in a desiccator until dry weights could be measured. The two larger lots, to be used for the flavonoid analyses, were placed immediately in 95 % ethanol and kept in this solvent. Such a procedure was followed to eliminate possible partial destruction of the flavonoid substances during drying (12).

EXTRACTION: The plant material stored in 95 % ethanol was extracted (Soxhlet) with additional portions of this solvent. The extracts of each plant part for each harvest date were combined, concentrated at reduced pressure, and made to a convenient volume (5 to 50 ml).

ANALYSIS: The analytical method used was that previously described (15). Although it should be regarded as semi-quantitative in nature, it was the only procedure which the author was able to apply with success to the mixture of flavonoids present in buckwheat seedlings. Aliquots of the extracts were spotted on strips of Whatman No. 1 filter paper 2.5 cm by 57.5 cm; these were equilibrated in a glass chromatographic chamber for 24 hours and then developed (descending) with the upper layer of the system chloroform–isobutanol–water (2–4–4 by volume). After drying, the strips were scanned at 350 mμ in a Beckman DU spectrophotometer. For each sample three replicate strips were run on different days, routine procedures being randomized. Quantities of rutin were calculated from observed areas under plots of absorbance vs. distance on chromatogram and from the previously obtained standard curve for rutin. Amounts of the four unidentified buckwheat flavonoids are expressed in arbitrary units since no standardization data for these substances were available. The use of such units enables quantitative comparisons to be made between samples for a given substance, although comparisons between substances would have no exact significance. Whenever the four unknown compounds may be identified and the proper area-amount constants can therefore be ascertained, these values will be readily convertible to more absolute quantitative measures.

RESULTS AND DISCUSSION

Data on seedling growth in terms of fresh weight and length are not reported here since changes in these measures were similar to those usually observed

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with such plant material. Dry weight values may be calculated from the data of table I, which embodies the results of the flavonoid analyses. The four unknown buckwheat substances are designated A to D (14). In order to emphasize the semiquantitative character of the analytical procedure, 90 % confidence

TABLE I

AMOUNTS PER PLANT AND PER MILLIGRAM DRY WEIGHT OF RUTIN AND OTHER FLAVONOID SUBSTANCES IN DEVELOPING BUCKWHEAT SEEDLINGS. RUTIN IN MICROGRAMS; OTHERS IN RELATIVE, ARBITRARY UNITS

DAYS AFTER GERMINATION	ORGAN	SUBSTANCE A		SUBSTANCE B		SUBSTANCE C		SUBSTANCE D		RUTIN	
		PER PLANT	PER MG DRY WT	PER PLANT	PER MG DRY WT	PER PLANT	PER MG DRY WT	PER PLANT	PER MG DRY WT	PER PLANT	PER MG DRY WT
	Etiolated seedlings										
0	Whole plant	<1	<0.1	<1	<0.1	<1	<0.1	<1	<0.1	3 ± 1	0.1
1	Whole plant	<1	<0.1	<1	<0.1	<1	<0.1	<1	<0.1	3 ± 1	0.1
2	Whole plant	<1	<0.1	<1	<0.1	<1	<0.1	<1	<0.1	4 ± 1	0.2
3	Root	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
	Hypocotyl	0	0.0	0	0.0	0	0.0	0	0.0	1 ± 1	0.7
	Cotyledon + pericarp	6 ± 3	0.2	5 ± 2	0.2	5 ± 1	0.2	6 ± 2	0.3	13 ± 6	0.6
	Whole plant	6	0.2	5	0.2	5	0.2	6	0.2	14	0.5
5	Root	0	0.0	0	0.0	0	0.0	0	0.0	1 ± 1	0.2
	Hypocotyl	0	0.0	0	0.0	0	0.0	0	0.0	2 ± 1	0.7
	Cotyledon	6 ± 3	0.6	6 ± 1	0.6	8 ± 1	0.8	8 ± 4	0.8	14 ± 2	1.4
	Pericarp	<1	<0.1	<1	<0.1	<1	<0.1	<1	<0.1	1 ± 1	0.2
	Whole plant	6	0.3	7	0.3	9	0.4	8	0.4	18	0.8
7	Root	0	0.0	0	0.0	0	0.0	0	0.0	1 ± 1	0.2
	Hypocotyl	0	0.0	0	0.0	0	0.0	0	0.0	4 ± 1	0.9
	Cotyledon	10 ± 5	1.4	10 ± 4	1.4	10 ± 3	1.4	16 ± 4	2.2	21 ± 7	3.0
	Pericarp	<1	<0.1	<1	<0.1	<1	<0.1	<1	<0.1	0 ± 1	0.1
	Whole plant	10	0.4	10	0.4	10	0.4	16	0.6	27	1.1
10	Root	0	0.0	0	0.0	0	0.0	0	0.0	0 ± 1	0.0
	Hypocotyl	0	0.0	0	0.0	0	0.0	0	0.0	7 ± 1	1.4
	Cotyledon	13 ± 5	3.2	13 ± 3	3.1	16 ± 5	3.8	20 ± 6	4.9	36 ± 8	8.9
	Pericarp	<1	<0.1	<1	<0.1	<1	<0.1	<1	<0.1	0 ± 1	0.0
	Whole plant	13	0.5	13	0.5	16	0.6	20	0.8	44	1.7
14	Root	0	0.0	0	0.0	0	0.0	0	0.0	1 ± 1	0.1
	Hypocotyl	0	0.0	0	0.0	0	0.0	0	0.0	9 ± 4	1.8
	Cotyledon	9 ± 4	2.8	11 ± 8	3.7	9 ± 4	2.8	18 ± 9	5.5	30 ± 14	9.3
	Pericarp	<1	<0.1	<1	<0.1	<1	<0.1	<1	<0.1	0 ± 1	0.0
	Whole plant	9	0.4	11	0.5	9	0.4	18	0.7	40	1.6
	Green seedlings										
10	Root	0	0.0	0	0.0	0	0.0	0	0.0	0 ± 1	0.1
	Hypocotyl	0	0.0	0	0.0	1 ± 1	0.2	0	0.0	8 ± 1	2.4
	Cotyledon	10 ± 8	1.5	8 ± 3	1.2	7 ± 6	1.0	12 ± 6	1.8	39 ± 30	5.9
	Pericarp	<1	<0.1	<1	<0.1	<1	<0.1	<1	<0.1	1 ± 1	0.1
	Whole plant	10	0.5	8	0.4	7	0.3	12	0.5	48	2.2
13	Root	0	0.0	0	0.0	0	0.0	0	0.0	1 ± 1	0.2
	Hypocotyl	0	0.0	0	0.0	1 ± 1	0.2	0	0.0	10 ± 4	2.6
	Cotyledon	11 ± 8	1.6	13 ± 4	1.9	20 ± 4	3.0	23 ± 6	3.5	46 ± 35	6.9
	Pericarp	<1	<0.1	<1	<0.1	<1	<0.1	<1	<0.1	1 ± 1	0.1
	Whole plant	11	0.5	13	0.5	21	0.9	23	1.0	58	2.4
22	Root	0	0.0	0	0.0	0	0.0	0	0.0	1 ± 1	0.1
	Hypocotyl	0	0.0	0	0.0	2 ± 1	0.5	0	0.0	11 ± 2	3.2
	Cotyledon	23 ± 11	3.0	19 ± 7	2.5	18 ± 10	2.4	25 ± 9	3.4	55 ± 25	7.4
	Epicotyl	0	0.0	0	0.0	0	0.0	0	0.0	12 ± 3	12.7
	Pericarp	<1	<0.1	<1	<0.1	<1	<0.1	<1	<0.1	0 ± 1	0.0
	Whole plant	23	0.8	19	0.7	19	0.7	25	0.9	79	2.8
27	Root	0	0.0	0	0.0	0	0.0	0	0.0	1 ± 1	0.1
	Hypocotyl	0	0.0	0	0.0	2 ± 1	0.3	0	0.0	18 ± 7	2.8
	Cotyledon	19 ± 9	2.9	20 ± 5	3.1	15 ± 3	2.4	27 ± 14	4.2	71 ± 52	11.0
	Epicotyl	0	0.0	0	0.0	0	0.0	0	0.0	12 ± 8	6.0
	Pericarp	<1	<0.1	<1	<0.1	<1	<0.1	<1	<0.1	0 ± 1	0.1
	Whole plant	19	0.5	20	0.6	17	0.5	27	0.8	102	3.0

limits are also presented where relevant. The widths of these confidence intervals should suggest the degree of caution with which the data must be approached. The interpretive discussion which follows is intended to suggest probable quantitative relationships in a very general way only. The author feels that the value of such generalities arises from the fact that no more exact information is at present available.

In the etiolated seedlings the amounts per plant of all five substances increased rapidly between the second and tenth days, then declined. In the case of whole plants amounts calculated on a dry weight basis showed a similar rise and fall; the dry weights themselves, of course, decreased throughout the period. Such values for single organs, especially the cotyledons, continued to rise between the tenth and fourteenth days for substances B and D and rutin; the opposite was true for substances A and C. Almost all of the total amounts of substances A, B, C, and D occurred in the cotyledons; certainly the changes in the cotyledons account for the quantities of these compounds which appeared during germination, since the pericarp levels remained approximately constant. Most of the rutin was also found in the cotyledons, but this glycoside was present in the other organs as well, increasing steadily in the hypocotyls.

A comparison of the 10-day values for green and etiolated plants reveals that in a rough way these are of the same order, perhaps indicating that the initial quantitative patterns in the two types of plant are not dissimilar. As previously noted (14), the only qualitative difference between green and etiolated plants was the occurrence of substance C in green hypocotyls. Until after the thirteenth day, however, the amount of C in the hypocotyls was only a small part of the total. This amount increased steadily, even though the total and that in the cotyledons appeared to decrease after the thirteenth day. A later decrease was also observed with substance A, while substances B and D increased in amount per plant and per unit dry weight throughout the experimental period. Rutin in the hypocotyls and cotyledons of green plants increased consistently, while quantities of this substance in the roots and pericarps remained essentially constant.

Since it was noted earlier that the ungerminated seed contains only one flavonoid—apparently rutin—in small quantities (traces of all five are present in the pericarps), the rapid appearance of each substance in the seedling must be accounted for in terms of its release from some bound form, synthesis de novo, or formation from some partially elaborated precursor. The present work offers no basis for decision on this point.

The cotyledons appear to be the most important seedling organs in the flavonoid picture. Apparently there is no movement of substances A, B, C (in etiolated plants), and D to other parts—or, if translocation does occur, destruction is immediate. Rutin and substance C (in green plants) are exceptional, but in these cases it is impossible to decide whether presence in several organs is a result of transport or of synthesis in situ.

A comparison of results for green and etiolated plants reveals no profound effect of light. This is in sharp contrast to the anthocyanin situation in buckwheat seedlings; light is known to be essential for the production of the latter pigment, although brief exposures will suffice (6, 9). Perhaps the biogenetic pathways for anthocyanin and other flavonoid substances are not closely associated in this plant.

Summary

Changes in approximate quantitative levels of rutin and four other buckwheat flavonoids have been followed in the several organs of developing green and etiolated seedlings. In both types of plant the amounts of all five substances increased rapidly after the second day of germination. Most of the total quantity of flavonoid material was localized in the cotyledons, although rutin was present in other organs of all plants and another substance occurred in the hypocotyls of green seedlings. It is suggested that in buckwheat seedlings the synthesis of anthocyanin may not be closely related to that of rutin and other flavonoid substances.

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Literature Cited

1. Badgett, C. O., Beinhart, E. G., Maher, J., and Connelly, J. A. Rutin content of several varieties of *Nicotiana rustica* and *N. glauca*. Arch. Biochem. **24**: 245–250. 1949.
2. Bate-Smith, E. C. Anthocyanins, flavones, and other phenolic compounds. In: Partition Chromatography, Biochem. Soc. Symposia (Cambridge, England) No. 3. 1949.
3. Couch, J. F., Naghski, J., and Krewson, C. F. Buckwheat as a source of rutin. Science **103**: 197–198. 1946.
4. Couch, J. F., Naghski, J., White, J. W., Sando, W. J., and Street, O. E. Tartary buckwheat as a source of rutin. U. S. Dept. Agr., Bur. Agr. and Ind. Chem. Mimeographed Circ. Ser. AIC222. 1949.
5. Hendrickson, R. and Kesterson, J. W. Hesperidin, the principal glucoside of oranges. Agr. Expt. Sta., Florida, Tech. Bull. **545**: 1–43. 1954.
6. Karstens, W. K. H. Anthocyanin and anthocyanin formation in seedlings of *Fagopyrum esculentum* Moench. Rec. trav. bot. neérl. **36**: 85–179. 1939.
7. Kawatani, T., Fujita, S., Ohno, T., and Katayanagi, M. Cultivation of buckwheat as a source of rutin. Jour. Pharm. Soc. Japan **73**: 837–841. 1953.
8. Kesterson, J. W. and Hendrickson, R. Naringin, a bitter principal of grapefruit. Agr. Expt. Sta., Florida, Bull. **511**: 1–35. 1953.
9. Kuilman, L. W. Physiologische Untersuchungen über die Anthocyane. Rec. trav. bot. neérl. **27**: 287–416. 1930.
10. McGregor, W. G. and McKillican, M. E. Rutin content of varieties of buckwheat. Sci. Agr. **32**: 48–51. 1952.

11. NAGHSKI, J., BRICE, B. A., and KREWSON, C. F. Production of buckwheat leaf meals with high rutin content. Amer. Jour. Pharm. 124: 297–306. 1952.
12. NAGHSKI, J., KREWSON, C. F., PORTER, W. L., and COUCH, J. F. Factors affecting the rutin contents of dried buckwheat meals. Jour. Amer. Pharm. Assoc., Sci. Ed. 39: 696–698. 1950.
13. PARIS, R. Rutin preparation from various species of buckwheat cultivated in France. Annales pharm. franç. 7: 21–26. 1949.
14. TROYER, J. R. The distribution of rutin and other flavonoid substances in buckwheat seedlings. Plant Physiol. 30: 168–173. 1955.
15. TROYER, J. R. Quantitative determination of buckwheat flavonoid substances by direct absorptiometry of paper chromatograms. Plant Physiol. 31: 71–74. 1956.

BRIEF PAPERS

VITAMIN C CONCENTRATION IN DEVELOPING AND MATURE FRUITS OF MANGO (MANGIFERA INDICA L.) [1]

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The mango (*Mangifera indica* L.) is an important tropical fruit, certain varieties of which have found increasing favor in temperate regions as dessert fruits. Previous studies have given the vitamin C (ascorbic acid) content of mature fruits from many varieties (5, 6, 7) as well as the distribution of vitamin C in the flesh (7). Although data from numerous other fruits are available, the seasonal pattern of vitamin C development in mangoes has not been reported, nor have analyses of the skin in relation to the remainder of the flesh. Information concerning the vitamin C content of the edible portion of these fruits, at different developmental stages and at maturity, seemed desirable.

Mango fruits at various stages of development were collected from mature trees of the varieties Amini, Mullgoa, Pico, and Turpentine (native) growing in the station orchards. All fruits of each variety were collected from single trees. The erratic flowering and extended period of fruit set permitted the collection of fruits of various ages at one time, thereby minimizing environmental variables. These fruits were apportioned among five arbitrary size classes, corresponding to developmental stages from approximately five weeks after fertilization to maturity. The age of each group was determined by comparing the average fruit size with growth data obtained by measuring the size of each variety at intervals from fruit-set through maturation. The reliability of this procedure has been established through a developmental study of the fruit (4). The fruits were prepared immediately after harvest for ascorbic acid assay. Fruits analyzed for vitamin C development had the skins cut away and the flesh removed down to the husks. Composite samples of the edible portion from 10 fruits of each class of the test varieties were prepared for triplicate analyses. Ascorbic acid maps of native and Amini fruits in different developmental stages were prepared by Barnett and Bourne's (2) method to show location of this substance.

Mature fruits analyzed for location of vitamin C were collected in the same manner as the samples of the younger stages and from the same trees, except that lack of material necessitated the use of Cambodiana in place of Pico. These mature fruits were peeled and cut up. Composite samples were made of five mangoes from each variety for 1) peelings, 2) outer mesocarp, 3) central mesocarp, and 4) inner mesocarp (adjacent to endocarp). All composite samples were analyzed by the diphenylhydrazine method of Schaffert and Kingsley (8), and the results are reported as mg/100 gm (mg %) of fresh mango flesh.

The vitamin C developmental patterns of the 4 mango varieties, Amini, Mullgoa, Pico, and Turpentine were similar. A marked decrease in the percent vitamin C of the flesh was noted between five weeks after fruit set and maturity (fig 1). Fruits of the smallest size class, average age about five weeks, yielded an average vitamin C value of 88 mg %, but at maturity (16 weeks) these same varieties averaged only 22 mg %. The greatest reduction in ascorbic acid concentration was noted between 5 and 10 weeks after fruit set. From about 10 weeks of age through maturity a more gradual decline, or none at all (Mullgoa) occurred. The vitamin C content determined for ripe Amini fruits, 11 mg/100 gm flesh, was about one half of the value reported previously; values of from 18.24 to 25.16 mg % have been recorded (6, 7). The ripe edible flesh of Cambodiana averaged less than 15 mg % vitamin C (table I) in this study, although Munsell (6) reported values of 30.52 and 37.50 for fruits of the same variety from this same orchard.

The ascorbic acid maps of native and Amini fruits showed a varying gradient from the sub-epidermal regions to endocarp. Maps made from mature fruits, although lacking the definition characteristic of the younger samples due to the decrease in vitamin C, demonstrated highest concentrations of ascorbic acid beneath the epidermis and adjacent to the endocarp. The low concentration of vitamin C in the central mesocarp tissue was confirmed by quantitative tests

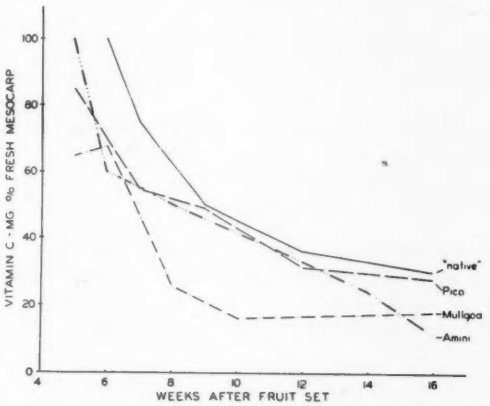

FIG. 1. Vitamin C content of the edible portion of 4 mango varieties during fruit development.

[1] Received September 29, 1955.

TABLE I

DISTRIBUTION OF VITAMIN C IN THE FLESH OF 4 VARIETIES OF RIPE MANGOES

VARIETY	TOTAL WT OF 5 MANGOES	PORTION OF FRUIT ANALYZED *							
		PEELINGS		MESOCARP					
				OUTER		MIDDLE		INNER	
		WT	VIT C	WT	VIT C	WT	VIT C	WT	VIT C
	gm	*gm*	*mg %*	*gm*	*mg %*	*gm*	*mg %*	*gm*	*mg %*
Amini	1095	187	22	261	15	194	10	238	12
Cambodiana	1532	257	28	261	20	279	10	418	10
Mullgoa	1669	264	26	348	11	397	16	428	20
Turpentine	868	118	29	176	7	211	14	217	18
Average	....	...	26	...	13	...	12	...	15

* Determinations are averages of 3 replications; maximum variation among replicates was less than 2 %.

on mature fruits of Amini, Cambodiana, Mullgoa, and Turpentine. Table I shows that the vitamin C content of the peels was very similar and was the highest found, averaging 26 mg %. The layer of flesh adjacent to the husk had the next highest average, 15 mg %. The central layers of mature mango fruits, as seen from the prepared maps, contained the lowest percentages of vitamin C, averaging about 12 to 13 mg %.

Although vitamin C content of developing mango fruits has not been recorded, there are reports on the effects of maturity on such contents of full-sized fruits. Some investigators have found an increase in vitamin C with maturation (1), and others have noted a decrease (3). Mustard and Lynch (7) found that mangoes picked when beginning to show color had lower ascorbic acid contents than either fully ripe or green mangoes. No significant differences were found by these same workers for vitamin C content of the various parts of ripe mango fruits, although they noted that the flesh of each individual fruit analyzed showed a decrease in ascorbic acid concentration with increased distance from the skin.

The minimum daily requirement for vitamin C for adults is about 30 mg. Since the central layers, or the edible portion, of mango contain approximately 13 mg % of this substance, about 2 to 3 ripe fruits per day would be sufficient to meet this requirement. Ripe mangoes of the varieties studied, therefore, appear to be fair sources of vitamin C.

SUMMARY

Vitamin C determinations made for this study on the developing fruits of mango demonstrated a downward trend common to the 4 varieties employed: Amini, Mullgoa, Pico, and Turpentine (native). This decrease in vitamin C concentration, from an average of 88 mg % to 22 mg %, was most marked in the period 5 to 10 weeks following fruit set; the final 4 to 6 weeks of fruit maturation was accompanied by little change in ascorbic acid concentration. Silver nitrate location maps and more refined tests by a diphenylhydrazine method showed that the highest concentration of vitamin C in ripe mangoes is not in the portion consumed. The highest concentrations are found in the peel and in the flesh adhering to the husks. The edible portion of mango fruits, however, appears to be a fair source of vitamin C.

LITERATURE CITED

1. BANERJEE, B. N. and ROMASORAMA, G. B. The vitamin A (carotene) and C content of mangoes. Agr. and Live-stock India 8: 253–258. 1938.
2. BARNETT, S. A. and BOURNE, G. Use of silver nitrate for the histo-chemical demonstration of ascorbic acid. Nature 147: 542–543. 1941.
3. HAWAIIAN AGR. EXPT. STA. Report, 1941–42. P. 134. 1943.
4. KENNARD, W. C. Development of the fruit, seed, and embryo of the Paheri mango (*Mangifera indica* L.). Bot. Gaz. (In press).
5. MILLER, CAREY O., LOUIS, L., and YANAZAWA, K. Vitamin values of foods in Hawaii. Agr. Expt. Sta., Univ. of Hawaii, Tech. Bull. 6: 1–47. 1947.
6. MUNSELL, HAZEL E. Ascorbic acid content of the mango in relation to variety. Food Research 11: 95–98. 1946.
7. MUSTARD, MARGARET J. and LYNCH, S. J. Effect of various factors upon the ascorbic acid content of some Florida-grown mangos. Agr. Expt. Sta., Florida, Bull. 406: 1–30. 1945.
8. SCHAFFERT, R. R. and KINGSLEY, G. R. A rapid, simple method for the determination of reduced, dehydro- and total ascorbic acid in biological material. Jour. Biol. Chem. 212: 59–68. 1955.

EFFECT OF MALEIC HYDRAZIDE ON PINEAPPLE[1]

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Flowering in pineapples is apparently auxin controlled (2) and may be induced by the application of various growth regulators or by temperature changes (3). It has been suggested that maleic hydrazide (MH), the well-known plant growth inhibitor, is an anti-auxin (1). It was of interest, therefore, to determine if flowering in a temperature sensitive plant could be delayed by MH and if the delay would be only temporary.

A field of healthy, mature Red Spanish pineapple plants was selected for this experiment. Treatments were made on December 6, before visible differentiation of flowers, and also on January 11, after differentiation had begun. The red coloration of the inner leaves was used as a criterion of flower initiation. Maleic hydrazide, as the diethanolamine salt, was dissolved in water at concentrations of 0.3, 0.03, 0.003, 0.0003, and 0.00003 % (active ingredient) and poured into the center of the plant at the rate of 10 or 20 ml per plant. There were 10 plants in each treatment. Flowering began about two and a half weeks after the experiment was initiated, and records were taken on December 26, January 10, and March 24. Eight out of 10 controls flowered by December 26; whereas none of those treated with 20 ml of 0.3 % MH had flowered. Six weeks later, 4 of these flowered, and the remaining 6 flowered at sporadic intervals thereafter. This was the only treatment which delayed flowering. The fruits from the plants delayed in flowering for 6 weeks were normal in size and shape, although they matured 6 weeks later than the controls.

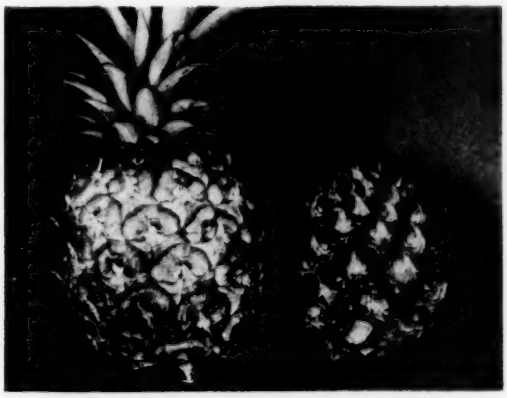

FIG. 1. Ripe fruit from control (*left*) and from plant treated after floral initiation with 20 ml of 1.0 % solution of the diethanolamine salt of maleic hydrazide (*right*). Crown growth was completely suppressed by this treatment, and the fruitlets failed to coalesce.

[1] Received October 6, 1955.

A second experiment was started on January 11, after flowering had begun. Since only the strongest solution used in the first experiment had any effect, this treatment, i.e., 20 ml of 0.3 % MH solution per plant was repeated, together with 40, 80, and 100 ml of the same solution poured into the center of the plant. No delay in fruit ripening was caused, but the fruits were distorted and decreased in size and weight as shown in figure 1. The individual fruitlets failed to coalesce, and extensive cork deposits were formed where the sides of the ovaries were exposed to the air. There was no apparent correlation with the amount of distortion and the amount of chemical applied. Development of the crown was suppressed in all treatments.

Percentage of fruits with gummosis[2] was very high in the treated plants of the second experiment, varying from 80 to 100 %. Fruits treated before flowering and untreated fruits had very little gummosis. This suggests that this disease may be physiological in origin.

These results show that flowering in response to low temperature in pineapple was temporarily inhibited by 0.3 % maleic hydrazide if the plant was treated about 20 days before flowering. Later treatment did not affect time of flowering or maturation but caused gummosis, distortion of the fruit, and suppression of crown growth.

LITERATURE CITED

1. LEOPOLD, A. C. and KLEIN, W. H. Maleic hydrazide as an antiauxin in plants. Science 114: 9–10. 1951.
2. VAN OVERBEEK, J. Control of flower formation and fruit size in the pineapple. Bot. Gaz. 108: 64–73. 1946.
3. VAN OVERBEEK, J. and CRUZADO, H. J. Notes on flower formation in the pineapple induced by low night temperature. Plant Physiol. 23: 282–285. 1948.

[2] This is an abnormal condition of the fruit in which a gummy exudate is secreted on the outside. The cause of this condition is not clear although both fungi and insects have been suggested as possible agents.

AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, *Annual Meeting, East Lansing, Michigan, September 1955:* The American Society of Plant Physiologists held its 30th Annual Meeting from September 5 to 8, 1955 on the campus of the Michigan State University. Our Society again cooperated with the Physiology Section of the Botanical Society of America in arranging the program. Two well attended symposia were sponsored. Dr. Walter Reuther acted as moderator for one, "The Process of Ion Accumulation by Roots"; and Dr. Harry Beevers presided at the second, "Respiration in Plants." In the 13 regular sessions, 153 contributed papers were presented—of this number, 32 had their origin in the Physiology Section of the Botanical Society of America, but 19 of these were from members of our Society.

At the annual banquet, the 31st anniversary of the Society was marked by an announcement of a Charles Reid Barnes Life Membership Award to Dean W. F. Loehwing and by an enthusiastically received Stephen Hales address by Professor Folke Skoog, who spoke on "The Chemical Basis of Growth Regulation in Plants."

Chairmen of the individual sessions held to the published schedule for presentation of papers. Much favorable comment has been received for this procedure. Only one person has objected to the secretary against its continuation. One of the session chairmen has protested vigorously about authors who were not present, did not deputize or submit any reason for not presenting their papers. Failure to notify the chairman of withdrawal had the effect of unduly breaking the program. Perhaps of still greater importance is the fact that the abstract becomes a part of the literature without a public hearing. It has been suggested this could become a serious matter, indeed.

Executive Committee Meeting, September 5th: The Executive Committee of the Society met September 5, 1955 at 8:00 P.M. in the Union Building of Michigan State University. Those present were Drs. A. S. Crafts, Chairman, K. V. Thimann, J. F. Stanfield, P. J. Kramer, B. S. Meyer, J. B. Hanson, C. A. Price, R. W. Howell, E. H. Toole, H. A. Borthwick, T. C. Broyer and A. W. Naylor.

Reports by the officers opened the meeting. Dr. Stanfield's report is as follows:

July 1, 1955

MEMBERSHIP:

Total members, July 1, 1954	1117
Renewals	4
New members	99
	1220
Resigned, dropped, deceased	67
	1158
Less those included in other groups	5
Total members, July 1, 1955	1153

SUBSCRIPTIONS:

U. S. subscriptions	for vol. 29, 372; for vol. 30, 387
Foreign subscriptions	for vol. 29, 775; for vol. 30, 845
Total subscriptions	for vol. 29, 1147; for vol. 30, 1232

SUMMARY OF CASH ACCOUNTS:

Checking Account, July 1, 1954	$14,904.44
Savings Account	9,500.00
Cash Balance, July 1, 1954	$24,404.44
Receipts, 1954–55	28,635.01
Total	53,039.45
Expenditures, 1954–55	23,502.18
Cash Balance, July 1, 1955	$29,537.27

Cash on hand, July 1, 1955

Checking Account	$15,537.27
Savings Account	14,000.00
	$29,537.27

SUMMARY OF ENDOWMENT ACCOUNT:
(Life Membership and General Endowment Fund)

Balance, July 1, 1954	$ 524.59
Income, 1954–55	
Barnes Life Membership Award	$ 100.00
From sales of back numbers	1,879.50
Excess Barnes and Hales	41.25
From investments	830.49
Three life membership purchases	300.00
	$ 3,675.74
Expenditures, 1954–55	
Purchase of three bonds	$ 3,015.00
Balance, July 1, 1955	$ 660.74

The Program Committee reported on costs of printing and mailing the abstracts. For the first time our abstracts are in a form which can be bound with the journal and since all members and subscribers have received a copy it may be expected that due respect will be given to the abstracts as an abbreviated form of publication. Authors should prepare their abstracts with as much care as the summary of a longer paper. Costs for the abstracts were as follows:

Preparation of manuscript copy	$ 0.00
Printing 2500 copies	856.50
Stamps	116.50
Express	1.85
Envelopes	30.00
	$1,004.85

Our representative on the A.I.B.S. governing board, Dr. P. J. Kramer, pointed out A.I.B.S. will require more money for operating expenses now that it is independent of the National Research Council. The new A.I.B.S. constitution was presented for consideration. It is quite similar to the old one. Most of the changes were changes necessitated by incorporation as a separate organization. Dues for member societies have been raised, in the case of our Society from

$800.00 to $1,000.00 annually. Dr. K. V. Thimann made the motion, seconded by Dr. B. S. Meyer that we ratify the new A.I.B.S. constitution. The motion was carried without dissent.

Following the reading of Dr. F. C. Steward's report as our representative to the National Research Council, it was voted that the concluding paragraph be read into the minutes. This paragraph is pertinent to the resolution adopted by the Society at its Annual Business Meeting, September 1954. It is thus stated:

"There was a special discussion of the problem of political clearance of recipients of research grants from the National Institutes of Health. Dr. Bronk in his capacity as an officer of the Academy and of the Research Council had energetically taken up this question with Mrs. Hobby. Dr. Bronk described his activities in this direction and referred 'off the record' to high level discussions of the problem which he had attended at the White House. While there is nothing very concrete to report, the Society may be interested to note that those present approved of a special motion of appreciation to Dr. Bronk for the way he is handling this problem in the interests of biologists and working through the National Academy."

Several vacancies soon to occur on the Editorial Board and committees were filled by appointment. Service is to begin January 1, 1956. Drs. Carl Leopold and C. H. Wadleigh were appointed to the Editorial Board of *Plant Physiology* for five-year terms; Dr. F. P. Cullinan was reappointed to the Board of Trustees for a three-year term; Dr. A. E. Schwarting was appointed Chairman of the Local Committee for the University of Connecticut meetings; Dr. H. A. Borthwick was asked to represent our Society on the Editorial Board of the *American Journal of Botany* for another year; and our new representative (3 years) on the AAAS Council is Dr. Marion Parker. Dr. Thimann requested relief from duty on the Committee on Professional Status and Training. A replacement is to be found.

Only one item of old business was discussed. This was the matter of re-publication of volumes 1 to 5 of *Plant Physiology*. At present three organizations have permission to reproduce it: Walter Johnson Reprint Corporation, New York, New York, J. S. Canner Micro-Card Company, Boston, Massachusetts, and University Microfilms, Ann Arbor, Michigan. It was the consensus that republication rights should not be extended to others if these companies make use of their privilege.

The first new business considered was the budget for *Plant Physiology* for the coming year. A budget of $20,000.00 was proposed by Dr. Thimann. Following approval of the motion, a discussion arose over possible new sources of revenue. Dr. Stanfield proposed, and it was approved, that the By-Laws be amended to read that the Life Membership be raised to $150.00 and the Patron Fee to $200.00. The new regulations are to take effect January 1, 1956.

Since Dr. Goddard has asked to be relieved of the editorship of *Plant Physiology*, a committee headed by Dr. K. V. Thimann was appointed to select a new editor. Recognition was made of the fine work Dr. Goddard has done for the Society and the hope expressed he can be prevailed upon to reverse his decision.

A motion was unanimously carried to meet with A.I.B.S. in 1958—probably at the University of Indiana in Bloomington.

The Midwestern plant physiologists presented a well drawn petition for consideration that their group be recognized as a section of the Society. Recognition of the new section was accompanied by dissolution of the Illinois, Purdue and Minnesota sections. Physiologists at Illinois and Purdue plan to continue operation on a seminar basis. Permission was granted the New England Section to change its name to the Northeastern Section. Henceforth this section will feel freer to invite physiologists outside the confines of New England to its meetings. The possibility and desirability of a strong Middle Atlantic States Section was discussed. In view of the growth in importance of the regional sections as a functional part of the Society, it was agreed thought should be given to furtherance in the development of the sections. Drs. Crafts, Borthwick and Broyer were constituted as a committee to draw up a brochure on sectional organization.

Concern was voiced over the relatively small number of practicing plant physiologists who are actually members of the Society. During a lengthy discussion it was suggested that more continuing members might be obtained by attracting larger numbers of graduate students into the Society one or two years before they graduate. A means to do this would be to reduce their dues. It was agreed Dr. Crafts should bring the matter before the general membership at the Annual Business Meeting.

As the final item of business the question was raised as to whether the new secretary, as Chairman of the Program Committee, should be instructed to follow the general procedure used this year in getting out the program. After a short but favorable discussion it was moved, seconded, and passed that "for next year (1956) we print and mail as we did this year."

Annual Business Meeting, September 8th: After acquainting those approximately 250 present with the actions taken by the Executive Committee the evening before and relating what had come of the Resolution made by the Society at Gainesville deploring the awarding of National Institutes of Health grants for research on political grounds, the meeting was opened for business.

President Crafts presented the problem of recruiting more members for the Society. Arguments in support of the idea of lower dues for graduate students were presented. Strong opinions were expressed pro and con. A vote was carried to institute a student category of membership. After some debate the fee for this membership was set at $5.00 with the suggestion each graduate student availing himself of this

privilege be certified by his Department Chairman at the time of paying dues.

The meeting was closed following a request by the membership that the Secretary convey greetings and best wishes to Dr. C. A. Shull, the Society's first president.—Respectfully submitted, AUBREY W. NAYLOR, Secretary.

AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, *Midwestern Section:* The first meeting of the Midwestern Section of the American Society of Plant Physiologists will be held at Iowa State College, Ames, Iowa, July 6 and 7, 1956. Sessions will include symposia, round-table discussions, and presentation of papers. Reservations of accommodations can be secured by writing Dr. Sam Aronoff, Botany Department, Iowa State College, Ames, Iowa.—JOHN B. HANSON, Secretary-Treasurer.

The difficulties encountered with the projection of lantern slides is a major source of irritation at the National Meetings. Mr. William G. Smith, biological photographer of the Boyce Thompson Institute for Plant Research, calls to our attention that certain standards have been established for the preparing of engineering and scientific charts for lantern slides.

These standards are fully explained in the booklet, "Engineering and Scientific Charts for Lantern Slides" (ASA—Z 15.1-1932, reaffirmed 1947). This may be purchased from the American Society of Mechanical Engineers, 29 West 39th Street, New York 18, New York, at a cost of ninety cents per copy.

Members of the profession are urged to obtain a copy of this booklet and see that their slides conform to the standards approved by the American Standards Association.—GEORGE L. MCNEW, Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York.

OUTLINES OF ENZYME CHEMISTRY—J. B. Neilands and Paul K. Stumpf. John Wiley & Sons, Inc., New York; Chapman and Hall, Ltd., London. 305 pages. 1955. $6.50.

This new textbook on the chemistry of enzymes will give much satisfaction to the biochemist and little comfort to the biologist. The first will be impressed by the fulness of outline and economy of detail; the second will be disappointed by yet another textbook which fails to recognize biology as anything but a handmaiden of chemistry. The point of view of the authors is easily perceived. They have treated enzymes largely as a special branch of chemistry and in so doing have given a very good account of the chemical and physical properties of enzymes. Wherever they have atempted to give a biological account of enzyme function the result leaves much to be desired.

The book is divided into four parts. Part 1 is entitled "General Principles." It begins with a hasty two-page introduction on the nature and history of enzymes. Since the book was written for students one cannot help wondering why no effort was made to present a broader philosophical background to the subject as a whole. But to raise this point of criticism is a little unfair for it touches upon the question of science education in a university, a question perennially debated and persistently unsolved. It is unlikely that this point will bother most instructors, for the book certainly has the merits commonly desired for academic courses. All points of general interest in enzyme behaviour are covered and the explanations provided are on the whole excellent. Principles of equilibria and ionization, isolation methods, criteria of purity, and characterization of enzymes are the general topics brought to the student's attention. The one criticism which might be raised from the didactic standpoint is that the different topics are unevenly treated. For example, much attention is paid to the elementary aspects of water dissociation, but in illustrating "the profound significance of ionization in enzymology" an understanding of the strongly basic character of the quaternary nitrogen atom and the weakly basic character of the tertiary nitrogen atom is largely assumed. In the isolation methods there is much advice which will prove useful to the laboratory worker, but the attempt to include all types of methods within 12 pages has resulted in very thin sketches. Many students will probably find the chapter on "Criteria of Purity" inadequate for it is hard to see how a student could learn anything about immunological methods in 5 lines; salt fractionation getting two pages, and electrophoresis two and a half. Indeed, Part 1 might have been a little expanded to relieve it of its encyclopaedic character.

Part 2 deals with the physical chemistry of the enzymes and includes the following: reaction rates, effect of substrate concentration on velocity, action of inhibitors, effect of temperature, effect of hydrogen-ion concentration, energetics, oxidation-reduction, enzyme-substrate compounds and specificity of enzymes. There is no doubt that this section is a most lucidly written one. The only item which might cause the reader concern is the long mathematical derivation of the effect of ionization on oxidation-reduction potentials; the detail seems out of balance with the rest of the treatment. It is regrettable that the chapter on specificity is so brief, occupying only three and a half pages.

Part 3 which deals with the classification of coenzymes, enzymes and respiratory enzymes, lacks the quality of the preceding section, probably because enzyme classification does better when approached from a biological standpoint. One cannot help thinking how much more colourful and exciting the treatment of iron enzymes would have been if an approach something akin to that of Granick in his article on "Inventions in Iron Metabolism" had been used.

Part 4 deals with "Metabolic Patterns" and covers metabolic processes, glycolysis, the hexose monophosphate shunt, the tricarboxylic acid cycle, oxidative phosphorylation, fatty acid oxidation and finally the synthesis of enzymes. In this section there is a major effort to link enzymatic activity with biological organization, but the link is disappointing. Thus one finds

the statement "biochemical examination of the cytological components of the cell has revealed an astonishing fact that a major portion of metabolic activity of the cell resides in small particles identified as mitochondria." Were the "fact" a fact it would indeed be astonishing but it simply is not so. Indeed the fact could be made even more astonishing by recognizing that some cells can lose these "small particles" and yet continue to live and multiply. The remainder of this section which deals with chemical transformations in the course of the various cycles listed is however competently written, and will prove helpful to every reader.

The final chapter on the synthesis of enzymes is quite out of tune. It does not even provide a formal completeness to the book. The student is asked to jump from fatty acid oxidation through genes to peptide bond syntheses, protein specificity, and into the intricate details of microbiological studies on enzyme adaptation. It is like throwing an obscure Hegelian passage into a German language primer. The book would be more graceful without it.

On the whole the book is a good one for students interested in the chemistry of enzymes. Its shortcomings are no more than those common to many university courses. Where to limit philosophical speculation so as to keep it from turning into a hollow scholasticism and factual information from turning into dull encyclopaedism is a difficult problem in education. It is unfortunate, however, that so often in so many places fact has been favoured to the exclusion of doctrine.—H. STERN, *Chemistry Division, Science Service, Department of Agriculture, Ottawa, Ontario, Canada.*

VEGETATION AND WATERSHED MANAGEMENT.—E. A. Colman. The Ronald Press, 15 East 26th Street, New York 10, New York. 412 pages. 1953. $7.00.

The author of this volume, which was sponsored by the Conservation Foundation, has been concerned with forest influences and watershed management for the last 25 years. This book is a successful attempt at synthesis and is concerned with water as a product of the land as well as with effects of vegetation in influencing water yield. Its objective is to review concepts and research in the relation of vegetation to water yield control so that an appraisal of existing knowledge may be considered together with possibly fruitful avenues for continued research.

The 16 chapters are presented in three sections blandly labelled 1) water yield and its control, 2) principles of water yield control, and 3) vegetation and water yield control.

An exceedingly brief first section defines the book's philosophical approach and is not markedly profound probably because of the author's desire to provide information at the beginning for those concerned with less technical aspects of water conservation.

Early in the second section Dr. Colman's enthusiasm for his field begins to emerge. He compares reservoirs and watersheds to suggest essential differences and similarities between them and their bearing on water yield control. With equal facility he appraises the influence of vegetation on hydrologic processes undergone by precipitation delivered to the land. There is also a treatment of how vegetation might be expected to influence the several aspects of water yield or how hydrologic processes, as modified by vegetation, could influence the high flows of streams, sediment load, and regimen and quality of water yield.

There is a final section of 8 chapters which is more lengthy than the other two sections combined. The subject matters here make for less evidence of continuity than was evident in the second section. Again a breadth of experience is revealed in the author's handling of chapters on vegetation regions, research, alternative kinds of vegetation, croplands, fire, grazed lands, tree-covered lands and finally a chapter on recapitulation.

Of special interest to this reviewer was the classification in five categories of pertinent research done in the past: studies designed to better understand hydrologic processes, those dealing in quantities involved in hydrologic processes, those in which different kinds of vegetation are compared with respect to water control, those seeking ways to correct damage to water yield processes by use or misuse of land, and vegetation change studies intended to improve water yield.

Dr. Colman concludes that undisturbed forest yields virtually no surface flow and erosion during storms and favors lowest storm-flow peaks in streams. Grass sod and other plants which provide dense, permanent soil cover also control runoff effectively while row and field crops show greatest water damage. In addition he points up the inadequacy of all rotation systems. Strip-cropping apparently has little or no effect in reducing surface flow as compared with rotation cropping.

The author believes that future research in hydrologic effects of fire should be designed to yield quantitative data under more carefully defined local conditions. Unquestionably burning promotes surface flow and erosion. Grazing, too, has deleterious effects. In the West heavy grazing motivated frequent flash floods, damage to lowlands, and reservoir sedimentation. In the East grazing promoted decreased ground cover and increased compaction which rendered soil susceptible to surface flow during storms.

The final chapter restates objectives and neatly summarizes: 1) Certain land uses and vegetation treatments have clearly defined hydrologic consequences. 2) There are only clues available of hydrologic effects of some land uses and vegetation treatments. 3) There is need for more research on hydrologic and physiological processes. 4) More research that will yield quantitative data applicable to entire watersheds is needed. 5) Further research is necessary to provide results that have quantitative application to problems of water yield control and such investigations will be time-consuming and costly. 6) Results of most research are safely and quantitatively applicable only

to areas similar to those where the research was conducted. 7) Considerable published information is available which can be advantageously analyzed.

Rather gingerly references to physiological processes seemed not to characterize adequately the opposing considerations of welfare of watershed vegetation and those of water yield control as viewed by hydrologists. In due fairness, however, the conflict is recognized, for instance, in the suggestion that evapotranspiration could be advantageously reduced by defoliation and the statement, "But considerable physiological research is necessary before we shall know when and under what conditions defoliation can be accomplished without unduly injuring the watershed vegetation."

Plant physiologists will be interested in the author's plea for research in evapo-transpiration related more closely to plant physiology, climate and soil under field conditions, so that the information obtained will have direct application in watershed management. In the light of the book's objectives the first part, at least, of the following statement puzzles this reviewer somewhat: "Studies are needed of physiologic processes in plants that affect transpiration, of root depths and rooting characteristics of plants of various kinds, and of the influence of soil water conditions and local weather conditions upon evapo-transpiration."

The author is thoroughly competent in his technical field and has carefully chosen his reinforcing data in appraising regional problems. His is a broad understanding of principles which is augmented with keen insight into variations in regional hydrologic situations. Furthermore, he is properly cautious about not extending local research findings too far.

Much could be gained in readability from considerable tightening up of the writing. There is a pronounced tendency throughout the book for recalling what was already covered in prior chapters and this is perhaps the most disappointing feature of Dr. Colman's work. Thus one finds, by way of only one of many examples, that on page 120 there are at least three such restatements which seem unjustified even in terms of any necessity for emphasis.

The format is quite acceptable although there is a tendency to neglect the freely quoted passages because they could be considered by some to be in unappetizingly small print. Especially lucid illustrations considerably enhance this informative contribution.—THEODORE T. KOZLOWSKI, *University of Massachusetts, Amherst, Massachusetts.*

PREPARATION OF MANUSCRIPTS FOR PUBLICATION IN PLANT PHYSIOLOGY

The following suggestions are made to authors writing papers for this journal in order to minimize revisions and editorial corrections. Though many points may appear arbitrary or trivial, attention to details is necessary to secure uniformity of format and style. The membership dues and subscription fees go almost entirely to publication costs, as all of our editors give their services without financial recompense. Hence the cooperation of authors in preparation of manuscripts minimizes expense and permits greater liberality as to length and number of papers published. Authors will find the following concise publications useful in preparation of manuscript copy: A. J. Riker, The Preparation of Manuscripts for Phytopathology. PHYTOPATHOLOGY XXXVI, pages 953–977, 1946; and S. F. Trelease, The Scientific Paper: How to Prepare it. Second Edition. Williams and Wilkins Company, Baltimore, Maryland, 1951.

1. Length. The allowable length of papers is 10 printed pages, with tables, figures, formulae and equations not to exceed 25 % of the total. Authors will be billed at cost for overrun and excess of figures and tables.

2. Format. In the preparation of typescripts, authors are requested to examine a January, 1956, copy of the journal of PLANT PHYSIOLOGY to note particularly the position of headings, general organization, punctuation, abbreviations and the methods of citing literature in order to bring the script into conformity with the general style of the journal. Leave a three inch margin at the top of the first page, liberal side margins and double spacing of lines to permit proofmarking and required editorial changes. Wherever possible metric units and centigrade temperatures should be used. If it is essential to use English units, such as in pounds per acre, include a conversion factor to metric units the first time the English units are used.

3. Headings. Leave all headings unscored and scientific names without underlining, as the editors will proofmark these items in accordance with the practice of the journal. Generic names used alone are usually capitalized but not italicized. In general, do not underscore any lines.

4. Footnotes. Avoid footnotes as far as possible by placing such items under acknowledgments or at end of summary. When footnotes are used, they should be numbered with Arabic numerals.

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